INTRODUCTION TO
PHYSIOLOGICAL CHEMISTRY

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BY

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PREFACE TO THE FOURTH EDITION

THE preparation of a new edition involves more than the mere task of recording new developments. It affords primarily an opportunity for the rearrangement, reintegration and rewriting of the subject matter in the light of existing knowledge. It imposes at the same time the obligation of retaining some measure of the historical perspective. As the solution to certain problems is reached there is often the tendency to lose sight of the painstaking labors and frustrations of those who laid the groundwork for the more recent achievements. For obvious reasons it has been impossible to dwell at length on the earlier contributions to every phase of the subject, but wherever possible an attempt has been made to include in the discussion sufficient material to enable the reader to gain some appreciation of the continuity of biochemical research and to realize the stages by which the present position has been attained.

It should be noted that in developing certain topics it has been possible to integrate the subject matter and to present it more comprehensively than in the earlier editions without increasing the length of the discussions. On the other hand this has not been possible in certain other instances, as in the study of enzymes, hormones, and vitamins, subjects in which there has been an extraordinary expansion during the last few years. The more recent developments in these fields not only have given us an insight into the chemical constitution of these classes of compounds, but also have brought out hitherto unsuspected relationships between them and have revealed the fact of their mutual and coordinated participation in the fundamental processes of life. To present these subjects adequately and coherently has necessitated a moderate allotment of additional space.

In former editions the subjects of digestion and the chemistry of enzymes were presented in one chapter. However, it has seemed desirable and appropriate to devote a separate chapter to each of these topics. In the revision for the present edition, the chapter on the chemistry of enzymes (Chapter VI) and the one on physiological oxidations (Chapter X) have been prepared by Dr. Oscar Bodansky.

The author is indebted to Dr. E. J. Cohn and the editors of *Physiological Reviews* for permission to reproduce Fig. 7 (page 110), to Dr. James

B. Sumner for the photomicrographs of crystalline urease and catalase, Figs. 9 and 12 (page 130); to Dr. John H. Northrop and the editors of the *Journal of General Physiology* for permission to reproduce Figs. 10, 11, 13, 15, and 16 (pages 130, 146, 147, and 150); to Dr. J. B. S. Haldane and Messrs. Longmans, Green and Company for permission to reproduce Figs. 17 and 18 (page 152); to Dr. Franklin Hollander and the editors of the *Journal of Digestive Diseases and Nutrition* for permission to reproduce Fig. 20 (page 170); to Dr. John P. Peters and his publisher, Mr. C. C. Thomas, for permission to reproduce Fig. 21 (page 190); to Professor F. J. W. Roughton and the editors of *Physiological Reviews* for permission to reproduce Fig. 29 (page 258); to Dr. E. Brand and the editors of the *Journal of Biological Chemistry* for permission to reproduce Fig. 33 (page 392); to Dr. Eugene F. DuBois for a photograph of the respiration calorimeter of the New York Hospital (page 512); and to Dr. W. C. Rose and the editors of the *Journal of Biological Chemistry* for permission to reproduce Figs. 40 and 41 (pages 568 and 570).

Valuable suggestions and advice have been received by the author from his friends and colleagues in other institutions, as well as from his associates at the University of Texas. He takes this opportunity to express to them his indebtedness. Grateful acknowledgments are also due to Miss Elizabeth Runge, Librarian of the University of Texas School of Medicine, for her work in verifying the references, and to the Misses Virginia B. Duff and Eddie Ball for their assistance in reading the galley proofs. Finally, the author wishes to express his appreciation to his publishers for their constant interest and for the care which they have exercised in the publication of this book.

MEYER BODANSKY.

April 20, 1938.

PREFACE TO FIRST EDITION

In aiding the student to correlate physiological chemistry with allied sciences and to define its scope, a textbook fulfils a very useful purpose. A small book, if it is sufficiently coherent and comprehensive, is likely to serve this purpose better than a large one, valuable as the latter may be as a source of reference. It was this idea that stimulated the author to write the present book. He has aimed to make it brief enough for use as an introductory volume and yet to give it sufficient scope to cover the field comprehensively. Laboratory methods and the description of tests have been omitted intentionally, since they are to be found in laboratory manuals devoted to the subject. The main aspects of physiological chemistry have been developed in relation to recent advances in the science. It is hoped that in this way the student will be afforded not only a knowledge of fundamental principles but also a realization of the developmental state of the subject.

It is obvious that a certain amount of condensation has been necessary, but the author hopes that he has not condensed the material at the expense of vital information. Wherever he has felt that collateral reading would be desirable, he has referred the student to easily accessible sources, such as journal articles, reviews, monographs, and other works. The student who enters upon the study of physiological chemistry is, strictly speaking, not a beginner. He is not unfamiliar with the principles of inorganic and organic chemistry, and in many cases he has received some training in physico-chemical concepts. He has therefore attained sufficient maturity to profit by collateral reading.

For a considerable amount of the material the author was dependent upon various books and journal articles. To the authors of these he takes this opportunity to acknowledge his debt of gratitude. *Physiological Reviews*, edited for the American Physiological Society and published by the Williams and Wilkins Co., Baltimore, Md., and the "Monographs on Biochemistry," edited by R. H. A. Plimmer and F. G. Hopkins and published by Messrs. Longmans, Green and Co., have been of especial value to the author in helping him to correlate and synthesize the vast literature on the subject of physiological chemistry. He also takes this opportunity to express his gratitude to Dr. Graham Lusk and

Dr. D. D. Van Slyke for their kind permission to reproduce certain material from their works. The author also wishes to thank P. Blakiston's Son and Co., for their kindness in permitting him to reproduce, from Hawk's "Practical Physiological Chemistry," the absorption spectra given on page 157 of this book.

In the preparation of the book, much discerning advice and criticism was received from Dr. C. L. Alsberg, director of the Carnegie Food Research Institute at Stanford University. The author takes this occasion to express his sincere gratitude for Dr. Alsberg's unfailing interest and almost daily encouragement.

Some of the points of view developed in the present volume the author has derived from his teacher and friend, Dr. William C. Rose of the University of Illinois. He wishes to acknowledge at this time his debt to Dr. Rose, as well as a similar debt to Dr. Byron M. Hendrix of the University of Texas.

Important suggestions were also received from the author's colleagues at Stanford University, among whom are included Professors L. B. Becking, George S. Parks, and Robert E. Swain.

For reading the proof and for valuable criticism, the author is indebted to Dr. B. M. Hendrix and Dr. Marion Fay of the University of Texas. He also wishes to thank Miss Elizabeth D. Runge, librarian of the University of Texas School of Medicine, for her assistance in verifying the references.

The author will at all times welcome suggestions and criticism.

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December 8, 1926.

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PHYSIOLOGICAL CHEMISTRY

CHAPTER I

INTRODUCTION

The subject of physiological chemistry, or biochemistry, is primarily concerned with the chemical aspects of life. Its rapid growth as a science has been intimately related to the development of theoretical chemistry, particularly in the fields designated as organic and physical. While biochemistry has drawn largely from these sources, it has in turn contributed fundamentally to every phase of biological science. We may illustrate this, for example, by the volume of experimental research summarized in such works as Czapek's "Biochemie der Pflanzen,"[1] or the more recent "Plant Physiological Chemistry" by Harvey.[2] Or we may consider in this light von Fürth's "Vergleichende chemische Physiologie der niederen Tiere,"[3] a classic prepared a generation ago, embodying the then accumulated data of the chemistry of digestion, respiration, nutrition, metabolism, excretions, secretions, tissue pigments, poisons, etc., of the lower forms of animal life—knowledge to which much has been added in the intervening years. And again there is the monumental work of Joseph Needham, "Chemical Embryology,"[4] which further testifies to the all-embracing influence that biochemical research has exercised in extending and amplifying biological science.

A considerable proportion of present-day research in bacteriology, immunology, and pathology either has a chemical basis or depends upon biochemical methods of study. But significant as have been the contributions of chemistry to these fields of investigation, their importance in illuminating many obscure problems of clinical medicine has been even more encouraging. Select at random a journal devoted to clinical investigation to ascertain the prevailing trend. Many of the contributions are concerned with such problems as calcium and phosphorus

[1] F. Czapek, "Biochemie der Pflanzen," Jena, 2d edition, 1913.

[2] R. B. Harvey, "Plant Physiological Chemistry," Century Co., New York, 1930.

[3] O. von Fürth, "Vergleichende chemische Physiologie der niederen Tiere," Jena, 1903.

[4] J. Needham, "Chemical Embryology," The Cambridge University Press, 1931.

metabolism, normal and pathological calcification, the colloid osmotic pressure of the blood, serum protein in relation to disease of the kidney, the relation of the adrenals to sodium and potassium metabolism, the normal and pathological variations of the concentration of lactic acid in the blood, the relation of protein intake to the course of renal disease, the composition of gastric juice and the factors which may alter it, etc. Examples like these may be multiplied many times by referring to the numerous journals, published in English and other languages and representing internal medicine, pediatrics, obstetrics, and even gynecology, surgery, and ophthalmology.

No boundaries actually separate physiology from biochemistry, or these from biophysics. The three subjects, at the present stage of development, are so closely interrelated, so mutually dependent on one another, that their classification as separate sciences is purely arbitrary. To a more limited degree, this interrelationship extends to pharmacology and pathology. Much space and emphasis, for example, are given in modern textbooks of physiology to chemical and physical concepts, and an even greater assimilation of this type of fundamental knowledge characterizes an ever-increasing proportion of the work published from physiological, pharmacological, and pathological laboratories. In short, the biochemist is close kin to the physiologist, pharmacologist, and pathologist, and in order that he may work in cooperation with them, should learn something of their methods and viewpoints.

The student of biochemistry should be equipped with a knowledge of fundamental chemical principles, as he must learn to apply these principles to the study of physiological processes. He also requires technique in quantitative chemical analysis. With this scientific equipment as a minimum, he may proceed to study the composition of tissues, the chemical constitution of foodstuffs, the fate of these in digestion and metabolism, the nature of enzymes and the ways in which they act, the data of animal calorimetry, and similar problems. It should always be borne in mind, however, that in the nature of things he will be limited in certain respects. A tissue subjected to chemical manipulation is no longer a living tissue; the process of analysis involves its alteration or destruction. One of the student's tasks, therefore, is to learn to correlate analytical data with function. As biochemists, we are especially interested in the numerous changes or reactions that occur in every living cell of the living organism.

That the cell is the unit structure of the living organism is a fundamental concept in biology. The cell is largely composed of protoplasm, a complex and heterogeneous mixture of substances, viscid in consistency, which was early recognized as the active living part of the organism and which Huxley characterized as the "physical basis of life."

It is customary to describe protoplasm as composed of water, protein, fats, carbohydrates, inorganic salts, enzymes, and other substances. But even if our knowledge of the organic constitution of protoplasm

were complete, it would still afford us no clear insight into its extraordinary properties. L. J. Henderson [5] has asserted that a sufficiently clear and intelligible definition of the term protoplasm is at present impossible. Conspicuous, however, is the interdependence of the various components of protoplasm and their apparent organization into coordinated physiochemical systems which underlie its manifold activities and properties. Significant also is the fact that in the same cell a number of reactions may occur simultaneously and in an orderly fashion and without interfering with one another, reactions of oxidation and reduction, hydrolysis and synthesis, upon which depend the irritability, nutrition, respiration, metabolism, growth, and self-perpetuation of protoplasm—properties which are conventionally cited by the biologist as the criteria that distinguish the living from the lifeless.[6]

Elementary Composition of Tissues. Water constitutes from 70 to 90 per cent of most tissues.[7] Accordingly, in a consideration of the elementary composition of protoplasm and tissues, it is obvious that both oxygen and hydrogen are quantitatively important. Indeed, the human body is more than 60 per cent oxygen and nearly 10 per cent hydrogen. Together with carbon, these elements enter into the constitution of carbohydrates, and with carbon, nitrogen, and sulfur into the composition of proteins. Phosphorus occurs in certain proteins and lipids and in inorganic combination with sodium, potassium, and calcium. The order of abundance in the human body of the four first-named elements is as follows: C, 20.2 per cent; N, 2.5 per cent; S, 0.14 per cent; P, 1.14 per cent.

Sodium and potassium are widely distributed in plants and animals and are very important physiologically. Potassium is more abundant than sodium in plants. Human blood plasma contains more sodium than potassium, whereas the reverse holds in the red corpuscles. The human body has been estimated to contain 0.10 per cent sodium and 0.11 per cent potassium.

Calcium is an essential cell constituent and is especially abundant in the skeletal structures of vertebrates. Magnesium is likewise a

[5] L. J. Henderson, "Blood," New Haven, 1928, p. 5.

[6] For a comprehensive view of the subject the student is referred to E. B. Wilson's "The Cell in Development and Heredity," Macmillan, New York, 1928, Chap. I; as well as to his review, "The Physical Basis of Life," in "Colloid Chemistry," edited by J. Alexander, Chemical Catalog Co., New York, Vol. II, 1928, pp. 515–524. Consult also in the same volume: W. Seifriz, "The Physical Properties of Protoplasm," pp. 403–450; L. V. Heilbrunn, "Protoplasm," pp. 451–459; R. S. Lillie, "The Colloid Structure of Protoplasm and Protoplasmic Action," pp. 461–466; R. Chambers, "The Nature of the Living Cell as Revealed by Micro-manipulation," pp. 467–486. It may also be added that a journal entitled *Protoplasma* is devoted almost exclusively to problems pertaining to the cell and to protoplasm, and that a series of monographs ("Protoplasma-Monographien") have appeared in recent years which afford authoritative reviews of the various aspects of the subject.

[7] Muscle contains about 75 per cent of water, bone about 40 per cent, and the enamel of the teeth, the hardest tissue of the body, approximately 5 per cent.

widely distributed constituent of tissues. It is a component of the chlorophyll molecule, chlorophyll being the green pigment of plants and a very important factor in plant economy. Marine algae contain both elements in large amount. The calcium content of the human body has been estimated to be 2.5 per cent; the amount of magnesium is about 0.07 per cent.

Iron is an essential constituent of plant and animal protoplasm. It is a constituent of hemoglobin, the respiratory pigment contained in the red blood corpuscles, as well as of certain tissue pigments that are even more widely distributed in nature than hemoglobin. The content of iron in the human body is estimated to be 0.01 per cent.

In certain of the lower animals, the Mollusca and Crustacea, copper-protein compounds (hemocyanins) are present and are said to play a rôle similar to that of hemoglobin in other forms of animal life. It has also been shown that copper, zinc, manganese, aluminum, and even nickel and cobalt are normal constituents of plant and animal organisms.

Certain marine organisms (corals, sponges) contain an iodine derivative of tyrosine, 3,5-diiodotyrosine. This compound is also present in the thyroid gland, which contains in addition the important iodine-containing substance thyroxine. Brain, liver, and other animal tissues are known to contain iodine, as well as small amounts of bromine. These elements likewise occur in many plants. Chlorine is the most abundant of the halogens, occurring largely in inorganic combination with sodium and potassium. The human body contains 0.16 per cent of chlorine. In small amounts, fluorine is widely distributed in nature, particularly in bones, teeth, and the shells of molluscs.

Silicon is found in plants and in many marine organisms, such as diatoms and sponges. In the higher animals, it has been found in hair, skin, lung, liver, kidney, muscle, thymus, and other tissues.

Among the more uncommon elements may be mentioned arsenic (found in minute quantities in the thyroid, brain, liver, hair, etc.), boron, lead (present in certain corals), and vanadium (found in the blood of *Ascidia*). Still other elements, including lithium, cerium, rubidium, barium, radium, strontium, and even gold, have been reported as present in living tissues, but whether those in the last group are normal constituents of protoplasm, or whether their occurrence is merely adventitious, it is at present impossible to say.[8, 9]

[8] In this connection the student is referred to a review by M. Swartz Rose, "Nutritional Significance of Some Mineral Elements Occurring in Traces in the Animal Body," *Yale J. Biol. and Med.*, **4**, 499 (1932). See also J. H. Sheldon and H. Ramage, "A Spectrographic Analysis of Human Tissues," *Biochem. J.*, **25**, 1608 (1931).

[9] Improved methods for the detection and estimation as well as for the localization of inorganic constituents in cells and tissues have been developed in recent years. One procedure depends on heating mounted tissue specimens in a red-hot furnace so as to burn away all the organic matter, leaving the mineral part of the tissue presumably in the position it occupied in the living cells. As a further develop-

The earthly origin of man is not a mere figure of speech, for all the elementary constituents of plant and animal organisms are to be found in the earth. But it is interesting to observe that, although there is an apparent abundance of plant life, the carbon content of the earth's crust is only about 0.18 per cent. In the human body, however, it is about 20.2 per cent. Next to oxygen, silicon is the most abundant element in nature; yet in our bodies it is present in an almost negligible amount. Aluminum is likewise plentiful in the earth, but is present in exceedingly small amounts in the human body.[10]

Relation to Composition of Sea Water. Comparative studies of the mineral constituents of tissue fluids have brought out the highly interesting fact that there is a remarkable uniformity of composition in different animals. A particularly extensive study has been made with respect to the elements sodium, potassium, calcium, and magnesium. In the following table are given the results obtained by Macallum with the blood sera of various animals, the results being calculated on the basis of 100 for the percentage concentration of sodium in any given species. In the table are also included analyses of ocean water. What is the significance of these observations? In the first place, it is to be noted that the proportion of calcium to sodium in sea water and in sera is nearly the same. The correspondence is not so close with potassium, but nearly so. On the other hand, there is considerable variation in respect to magnesium. Macallum and, somewhat earlier, the German physiologist, Bunge, suggested that the high content of sodium chloride

TABLE I

COMPOSITION OF SERA OF ANIMALS AS RELATED TO THAT OF SEA WATER*

Species	Na	K	Ca	Mg
Dogfish (*Acanthias vulgaris*)	100	4 6	2 7	2.5
Cod (*Gadus callarias*)	100	9.5	3.9	1.4
Pollock (*Pallachinus virens*)	100	4 3	3 1	1.5
Dog	100	6.9	2.5	0.8
Man	100	6 1	2.7	0.9
Lobster (*Homarus americanus*)	100	3.7	4.9	1.7
Horseshoe crab (*Limulus polyphemus*)	100	5.6	4.1	11.2
Jellyfish (*Aurelia*)—tissue fluid	100	5.2	4.1	11.4
Ocean water	100	3.6	3.9	12.1

* After Macallum, *Trans. Coll. Phys. of Philadelphia*, **39**, 289, 1917.

ment in technique, the organic material in a given area is incinerated by an electric spark and the emission spectrum photographed in order to reveal the inorganic constituents present. For details the reader is referred to A. Policard's paper, "Some New Methods in Histochemistry," Harvey Lectures, Series **27**, p. 204, 1931-32.

[10] A comparison of the composition of the human body with that of the earth's crust is given by A. J. Lotka, "Elements of Physical Biology," Williams & Wilkins, Baltimore, 1925, p. 192.

in the blood of vertebrates may be an inheritance from our remote ancestors who lived in the sea. Supposing that these animals took to the land after the development of a closed circulatory system, it might follow that the composition of the sea, as it was at that time, has persisted in their blood to the present day. How, then, are we to account for the divergences in potassium and magnesium? It has been suggested that since the Cambrian period less potassium has been supplied to the sea than prior to that time, because so much of this element has been required by plant life, which has been more profuse since the Cambrian era. However, the magnesium content of the ocean and the proportion of magnesium to sodium have been steadily increasing since pre-Cambrian time, the concentration found at present in the higher animals corresponding presumably to the low magnesium content of the sea at the time the animals in question acquired a terrestrial habitat. The calcium content has been increasing but slowly, owing perhaps to the utilization of calcium in the building of corals and the bones and shells of other marine organisms. To sum up Macallum's theory, it may be stated that the blood serum of mammals resembles, except for the difference in its magnesium content, diluted sea water of our own day.[11]

Baldwin [12] has analyzed Macallum's argument, but finds no cause for surprise over the fact that the bloods of different animals resemble one another so closely, for it could not have been otherwise. He states: "the composition of the blood has remained the same because the conditions under which life is possible have remained the same. It has remained so by no static process like the kind of fossilization supposed by Macallum; it has been actively maintained, as indeed it must if life were not to become extinct."

Properties of Solutions, Relation to Gas Laws. The behavior of substances in solution occupies a dominant position in biochemical and physiological thought and discussion. Nor can it be otherwise when we consider that water and the dissolved substances which it contains constitute the "internal environment" of the animal organism. It is a curious fact, and one with which the student is doubtless familiar, that substances in solution behave very much like gases. For this reason, a brief review of the gas laws is appropriate in this connection.[13]

[11] For fuller details the student is referred to A. B. Macallum, "The Paleochemistry of the Body Fluids and Tissues," *Physiol. Rev.*, 6, 316 (1926); see also F. W. Clarke, "The Data of Geochemistry," 4th edition, *U. S. Geol. Survey, Bull.* 695.

[12] E. Baldwin, "An Introduction to Comparative Biochemistry," Cambridge University Press and Macmillan Company, 1937, p. 15.

[13] The scope of this book does not permit a lengthy and detailed consideration of physico-chemical principles. All that can be attempted here is a brief résumé of a few salient facts and the definition of certain terms commonly encountered in biochemical discussions. The need for a more comprehensive understanding and appreciation of the fundamental facts of physical chemistry by the student of biology and medicine has been so generally recognized that several splendid books have been

Gas Laws. The relations governing the behavior of gases were first discovered empirically and, as later found, only approximately. *Boyle's law* (1662) states that, when the temperature of a gas is held constant, the volume varies inversely as the pressure:

$$v \propto \frac{1}{p} \text{ (temperature constant)} \tag{1}$$

Gay-Lussac's law (1801) states that, when the pressure is held constant, the volume varies directly as the absolute temperature:

$$v \propto T \text{ (pressure constant)} \tag{2}$$

A corollary of the first two laws is that, at constant volume, the pressure of a gas is directly proportional to its absolute temperature.

$$p \propto T \text{ (volume constant)} \tag{3}$$

The expressions (1) and (2) may be combined to give (4):[14]

$$v \propto \frac{T}{p} \tag{4}$$

and, introducing a proportionality factor,

$$v = k\frac{T}{p} \tag{5}$$

or

$$pv = kT \tag{6}$$

The value of k may be found by substituting for p the standard pressure of 1 atmosphere; for v, the volume of the molecular weight of the gas in grams; and for T, the standard temperature, 0° C., or 273° absolute. Using the proper units, k, which we will call the *molecular gas constant* and designate by R, becomes 0.08204 liter-atmosphere per degree, or 8.31×10^7 ergs per degree.[15] Since, by *Avogadro's law*,

written with this specific purpose in mind, and these books the student is urged to consult. See, for example, D. I. Hitchcock, "Physical Chemistry for Students of Biology and Medicine," C. Thomas, Springfield, Ill., 2d edition, 1934.

[14] The mathematical principle underlying this derivation is stated by Hall and Knight as follows: "If A varies as B when C is constant, and A varies as C when B is constant, then will A vary as BC when both B and C vary." This proposition "can easily be extended to the case in which the variation of A depends upon that of more than two variables. Further, the variation may be either direct or inverse." (For details, see H. S. Hall and S. R. Knight, "Higher Algebra," Macmillan, 4th edition, 1929, pp. 23–25.)

[15] $R = \frac{(1 \text{ atmosphere})(22.4 \text{ liters})}{273} = 0.08204$ liter-atmosphere, per degree.

In the c.g.s. system of units, $R = \frac{(76 \text{ cm. Hg pressure})\ (22{,}400 \text{ cc.})}{273°}$. A pressure of 76 cm. Hg is equal to a force of $76 \times 13.6 \times 980$, or 1.013×10^6 dynes per $\overline{\text{cm.}}^2$ Therefore,

$$R = \frac{\left(\frac{1.013 \times 10^6 \text{ dynes}}{\overline{\text{cm.}}^2}\right)(22{,}400\ \overline{\text{cm.}}^3)}{273°} = 8.31 \times 10^7 \text{ ergs, per degree}$$

the molecular weight of all gases occupies the same volume at a given temperature and pressure, the relation $pv = RT$ will hold for all gases. The equation naturally applies to quantities of a mole when the proper factor is introduced. Hence, dropping subscripts and introducing this factor, we obtain the general formulation of the three gas laws:

$$pv = nRT$$

when n denotes the number of moles of gas present.

The above relation, $pv = nRT$, is approximate, holding for certain gases only within certain temperature and pressure ranges. When it is attempted to give this relation a theoretical background, as in the kinetic molecular theory, it is necessary to assume (1) that the molecules of a gas are so far apart that they exert no attraction upon one another and (2) that the space which they themselves occupy is negligibly small in comparison with the volume of the containing vessel. These are conditions attained by no real gas and hence may be taken as properties of the ideal or perfect gas. In chemical thermodynamics, compliance (1) with the relation $pv = nRT$ and (2) with the relation that the energy is a function of the temperature alone is taken as the definition of a perfect gas.

Osmotic Pressure. Of the many properties which substances in solution exhibit, one of the most interesting is that of osmotic pressure. The classical experiments of Pfeffer may be used to illustrate the phenomenon. The accompanying sketch (Fig. 1) shows the form of Pfeffer's apparatus. A precipitate of copper ferrocyanide is deposited in the walls of a porous cup A. B is a mercury manometer. The cup is filled with the solution to be tested, the surface of the latter being made even with the surface of the mercury in C. Tube D is then sealed off and the whole apparatus immersed in a bath of distilled water kept at constant temperature. The water at first passes through the membrane, increasing the pressure inside the apparatus and forcing the mercury up the manometer tube. This continues until a point is reached when there is no further increase in pressure in the cup. The reading of the manometer is then taken as the osmotic pressure of the solution.

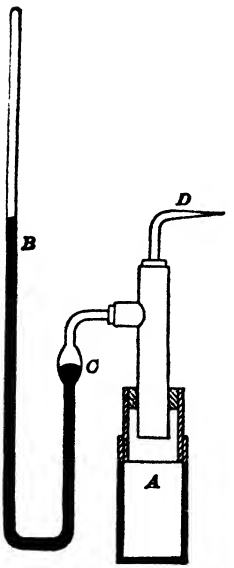


FIG. 1.

Before considering the mechanism of this phenomenon, let us see what relation it bears to the concentration of the solute and the temperature of the solution. The following data from Pfeffer show that the osmotic pressure is directly proportional to the concentration.

This relation, the importance of which was first pointed out by van't Hoff, may be put into a slightly different form. In making up

TABLE II

RELATION OF OSMOTIC PRESSURE TO CONCENTRATION

C Per Cent of Cane Sugar	P cm. Hg	$\frac{P}{C}$
1	53.5	53.5
2	101 6	50.8
4	208 2	52 1
6	307.5	51.3

solutions of a definite amount of material, the concentration of the solution varies inversely as the volume of the solution. For instance, 2 grams dissolved in 100 cc. gives twice as strong a solution as the same amount dissolved in a volume twice as great, 200 cc. Hence, if the osmotic pressure of a solution is directly proportional to the concentration of the solute, it is inversely proportional to the volume.

$$\text{Volume} = k_1 \frac{1}{\text{pressure}}$$

This relation is analogous to Boyle's law for gases.

Similarly, as the following table indicates, the relation between osmotic pressure and temperature is analogous to Gay-Lussac's law, the pressure varying directly with the temperature.

TABLE III

VARIATION OF OSMOTIC PRESSURE WITH TEMPERATURE

Temperature °C	T Absolute Temperature 273° + °C.	Osmotic Pressure in cm. Hg	$\frac{\text{Osmotic Pressure}}{T} = k_2$
6 8	279.8	50.5	0 181
13.2	286.2	52.1	0 182
14.2	287 2	53.1	0 185
22 0	295 0	54.8	0.186
36 0	309 0	56 7	0.183

The question that next presents itself is whether Avogadro's hypothesis applies to solutions. The significance of Pfeffer's work was not appreciated until van't Hoff recalculated the data in order to bring out the existing relations between pressure, volume, and temperature. In studying the effect of temperature on osmotic pressure, Pfeffer used a 1 per cent solution of cane sugar (1 gram in 100.6 cc. of solution) or one

containing 0.02906 mole of sucrose per liter. From the gas laws, van't Hoff calculated the gas pressure of 0.02906 mole of hydrogen at 0° C. and found it to be 0.649 atmosphere. Since, according to the formula, $P = k_3T$, the pressure of the gas would increase $\frac{1}{273}$, or 0.00367, for each degree about 0° C., or 273° A., then, for any other temperature, the pressure would be

$$0.649\ (1 + 0.00367t)$$

where t is the temperature in Centigrade. Van't Hoff now recalculated Pfeffer's data on this basis, comparing the observed results of osmotic pressure with the values computed for the various temperatures from the gas laws. These data are tabulated below:

TABLE IV

Temperature, °C.	Observed Pressure in Atmospheres	Calculated Gas Pressure in Atmospheres
6 8	0.664	0.665
13 7	0 691	0 681
15 5	0 684	0.686
22 0	0 721	0 701
32.0	0 716	0.725
36 0	0 746	0 735

For gases, R, the molecular (or molar) gas constant, in the equation $PV = RT$ is equivalent to 0.08204 liter-atmosphere. Taking 0.649 as the osmotic pressure of 1.0 per cent sucrose at 0° C. and 760 mm. Hg pressure, and 34.2 liters as the volume occupied by one mole of the solute, van't Hoff obtained 0.0813 as the value for R when the gas equations were applied to the solution. These results led him to the important conclusion that *the osmotic pressure exerted by any substance in solution is the same as it would exert if it were a gas in the same volume as that occupied by the solution.* All this holds in dilute solutions where the volume of the solute is small as compared with the volume of the solvent.[16]

[16] The laws of dilute solution which we have been outlining above are not detached pieces of information. They can be shown to follow rigorously, i.e., mathematically, from the First and Second Laws of Thermodynamics. The First Law is familiar to us as the *Law of Conservation of Energy.* Whenever a system undergoes a change in energy, the change is evidenced in the heat absorbed from or given off to its environment and the work done by or on the system. The Second Law, known as the *Law of Entropy,* is more difficult to understand. Ordinary observation shows us that every system, left to itself, changes in such a way as to approach a definite final state of equilibrium. Substances diffuse from concentrated solutions to dilute ones, heat passes from hot bodies to cold, clocks run down. In short, systems lose their capacity for spontaneous change. And they can lose this capacity without losing any energy. For instance, let us conceive of a system in which there is a hot

The formula $PV = nRT$, known as the van't Hoff equation, applies only to certain substances, such as urea, glucose, sucrose. Van't Hoff pointed out that the osmotic pressure of salts of strong acids and strong bases was greater than would be expected from this formula. How was this to be explained? In 1887 Arrhenius advanced the hypothesis that the molecules of certain substances when brought into solution dissociate into electrically charged particles or ions. This is known as the *theory of electrolytic dissociation.* It will be recalled that, many years before, Faraday classified substances as electrolytes and non-electrolytes; the former, he believed, were broken up by the passage of the electric current. What really happens, however, is that the molecules dissociate spontaneously and the ions carry the electric charge. The osmotic effect of a salt that dissociates almost completely is nearly twice as great as that produced by an equimolecular quantity of a non-electrolyte like urea or cane sugar. This is so because each molecule of NaCl, for example, may yield two particles or ions.

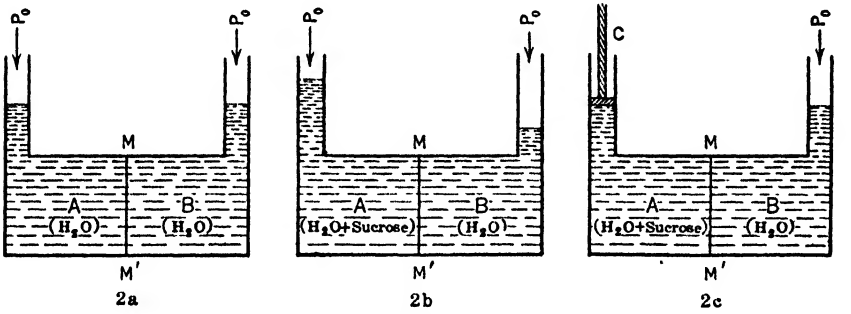


FIG. 2.

To illustrate further the meaning of the laws of dilute solutions and of van't Hoff's equation, in the case of osmotic pressure, suppose that we have a cell with two compartments, each containing distilled water and separated by a membrane MM' through which the water can pass (Fig. 2a). In this case the tendency of the water molecules to escape from A into B will be equal to the tendency for them to pass from B into A. As a result, no water passes either way. Suppose, now, that some soluble substance such as sodium chloride or sucrose which does not pass through the membrane is added to A. The addition of a solute lowers the escaping tendency of the solvent. Hence the escaping tendency of the water in A is now lowered whereas that in B, remain-

body and a cold one. The heat passes from the former to the latter, until finally the two are of the same temperature. No energy has been lost, but the capacity for spontaneous change has vanished. The more a system lacks the capacity for spontaneous change, the more *entropy* it is said to have. For a detailed discussion the student is referred to G. N. Lewis and M. Randall, "Thermodynamics," McGraw-Hill Book Co., New York.

ing the same, exceeds it. Water, therefore, tends to pass into compartment A and the level in A tends to rise. However, a sufficient pressure, P, exerted on the solution in A by means of piston C will prevent an actual passage of water. This pressure, P, *minus* the original (usually atmospheric) pressure, P_0, on the pure solvent is known as the osmotic pressure. The equation relating the osmotic pressure to other variables is:

$$\frac{d(P - P_0)}{dN_2} = \frac{RT}{\overline{v_1}}$$

where N_2 is the mole fraction of the solute; $\overline{v_1}$, the partial mole volume of solvent; R, T, P, P_0, the meanings we have already assigned. In very dilute solutions this equation reduces to van't Hoff's familiar $(P - P_0)V = nRT$, or $pv = nRT$ (p. 8).

Elevation of the Boiling-point. The vapor pressure of a liquid increases with temperature. When the vapor pressure of the liquid equals the pressure of the atmosphere above it, the liquid boils. If a non-volatile substance is added, the vapor pressure is decreased. Hence it takes a higher temperature to produce a vapor pressure equal to the atmospheric pressure, that is, to make the liquid boil. The elevation in boiling-point produced by dissolving one mole (the molecular weight in grams) of a substance in 1000 grams of solvent may be termed the molar elevation of the boiling-point. It follows, therefore, that the osmotic pressure of a solution is directly proportional to the elevation of the boiling-point above that of the pure solvent. The osmotic pressure exerted by a molar solution of a non-electrolyte (molecular weight of a substance dissolved in a liter of water) is 22.4 atmospheres. Such a solution exhibits a boiling-point elevation of 0.54° C.

Depression of the Freezing-point. Biological fluids do not ordinarily lend themselves to boiling-point methods for determining osmotic pressure.[17] Since a substance in solution lowers the freezing-point of the solvent, freezing-point or cryoscopic methods have found wide application. Water freezes at 0° C. A molar solution of a non-electrolyte in water lowers the freezing-point of the water to −1.86° C. The freezing-point of mammalian blood serum is about −0.56°, corresponding to a 0.3 molar solution of a non-electrolyte, or about 0.16 molar NaCl, and equivalent to an osmotic pressure of 6.6 atmospheres. The freezing-point depression (Δ) of urine is much greater, being frequently in excess of 2.0°. Sea water freezes at about −2.3°. The Δ of the body fluids of coelenterates, echinoderms, worms, and crustaceans is about the same as that of the ocean water in which they live, namely 2.2–2.3.

[17] A. V. Hill has described a thermal method for measuring vapor pressure (*Proc. Roy. Soc. (London), A.*, **127**, 9 (1930); also described by R. Margaria, *J. Physiol.*, **70**, 417 (1930) and by A. V. Hill in "Adventures in Biophysics," University of Pennsylvania Press, Philadelphia, 1931, Chap. 1). The method is applicable to biological problems and has been found to have a high degree of sensitivity and accuracy.

This is also true for body fluids of the elasmobranchs, but not for the fluids of marine teleosts, which have a lower osmotic pressure.

Osmotic Pressure Phenomena in the Organism. DeVries made the first attempts to measure osmotic pressure in living cells, using the epidermal cells of certain plants for this purpose. The outer wall of most plant cells consists of a framework or skeleton of cellulose. In the normal state, the protoplasm within the cells presses closely against this framework. Placing a plant cell in solutions of higher osmotic pressure than that of the cell sap results in loss of water from the cell; the protoplasm contracts and draws away from the outer membrane. Owing to its rigidity, the cellulose wall can withstand considerable changes of internal pressure. The phenomenon described is called *plasmolysis*. The degree of plasmolysis is determined by the concentration of the outer fluid. If the plasmolyzed cells are placed in solutions of lower osmotic pressure than that of the cells themselves (i.e., *in hypotonic solutions*), they will regain their former appearance and turgor. In a solution of equivalent osmotic pressure to the cell contents (*isotonic solution*), plasmolysis does not occur. The osmotic pressure of cells of unknown tonicity can be determined by suspending them in a series of solutions of known concentration and observing the concentration of the solution that just fails to cause plasmolysis. Conversely, cells of known tonicity may be employed in determining the osmotic pressure of unknown solutions.

The membrane surrounding red blood corpuscles cannot withstand great changes of internal pressure. For this reason, when red corpuscles which are approximately isotonic with 0.95 per cent NaCl are immersed in hypotonic solutions, they swell, and, if the swelling is sufficient, the cells may burst. The process, called *hemolysis*, consists in the liberation from the corpuscles of hemoglobin, the red coloring matter of the blood. On the other hand, red corpuscles contract when placed in solutions of higher osmotic pressure (hypertonic solutions), the membrane acquiring a characteristic irregular or *crenated* appearance. The behavior of red corpuscles toward solutions of varying concentration has been made the basis of a method for determining indirectly the osmotic pressure of solutions. It involves the use of the hematocrit, which is a graduated capillary tube of small diameter into which blood may be drawn. On centrifuging at high speed, the corpuscles separate from the serum and collect at one end of the tube, the volume occupied by the corpuscles being read off from the graduations on the tube. When immersed in an isotonic solution, the corpuscles do not change in volume; but when suspended in hypotonic or hypertonic solutions, they increase or diminish in volume, as the case may be.[18]

[18] Osmotic changes in red blood cells may be determined by various other methods. It has been generally assumed that the red corpuscle takes in water without losing any osmotically active substances to the surrounding medium and that it thus acts as a "perfect osmometer." Actually the cells do not swell in hypotonic solutions as much

The ability of the organism to exist in the face of unusual changes of the *external* environment depends on the relative constancy of its *internal* environment.[19] This is illustrated by the osmotic pressure of the blood and tissues, the maintenance of which within certain narrow limits is of crucial importance. Most organisms contrive to achieve this end by one mechanism or another.[20] There are exceptions, however. Such a marine invertebrate as the spider crab, when placed in diluted sea water, loses salt from its body until the salinity of the internal environment is approximately the same as that of the outside fluid. Another exception is the dogfish, an elasmobranch, the blood of which has an osmotic pressure equivalent to that of the sea water in which it lives. If the environment is changed to diluted sea water (not below 75 per cent of the initial concentration), the osmotic pressure of the blood changes correspondingly, the adjustment being due to the diffusion of water into the animal, as may be shown by the increase of its weight. If the concentration of the sea water is still further diminished, the dogfish dies. Obviously, it does not possess the mechanism for maintaining its own internal environment, independently of the external environment. Other organisms possess this ability to a varying degree. The conger eel, for example, can tolerate a 1:10 dilution of the sea water which is its natural habitat, with only a moderate change in the osmotic pressure of the blood. The shore crab, though normally in osmotic equilibrium with sea water, is capable of withstanding marked changes in the concentration of its environment with correspondingly smaller changes in

as would be expected if this were true. J. Macleod and E. Ponder, *J. Physiol.*, **77**, 181 (1933).

[19] Claude Bernard's concept of the rôle of the internal environment is stated in his "Leçons sur les phénomènes de la vie," Paris, 1878, pp. 112–113. In translation (after Fulton, "Readings in Physiology," Thomas, Springfield, Ill., 1930, p. 308), it reads as follows:

"The living organism does not really exist in the *milieu extérieur* (the atmosphere if it breathes, salt or fresh water if that is its element) but in the liquid *milieu intérieur* formed by the circulating organic liquid which surrounds and bathes all the tissue elements; this is the lymph or plasma, the liquid part of the blood, which in the higher animals, is diffused through the tissues and forms the ensemble of the intercellular liquids and is the basis of all local nutrition and the common factor of all elementary exchanges."

According to the speculations of Claude Bernard, the primary object of all vital mechanisms, however varied they may be, is that of preserving the internal environment. To the extent that the constancy of the latter is maintained the organism becomes independent of its surroundings.

W. B. Cannon, professor of physiology at Harvard, introduced the term *homeostasis* to describe the coordinated physiological processes whereby most of the steady states of the organism are maintained. The preservation of the volume of the fluid matrix and the constancy of its composition (water, salts, sugar, calcium, protein, *p*H, etc.), the maintenance of a constant body temperature, and the natural defenses of the organism are examples of "physiological homeostasis." See W. B. Cannon, "Organization for Physiological Homeostasis," *Physiol. Rev.*, **9**, 399 (1929); "The Wisdom of the Body," W. W. Norton & Co., New York, 1932.

[20] E. Baldwin,[13] Chapter 2.

the osmotic pressure of its body fluids. So far as we know, the osmotic pressure of the blood of vertebrates is practically independent of the external environment.[21]

This does not imply, however, that the osmotic pressure is fixed and invariable; even in man it is subject to change within certain limits. Employing the method of measuring vapor pressure developed in A. V. Hill's laboratory, Margaria [22] determined the osmotic pressure of the blood of 18 men at rest and obtained an average value corresponding to 0.9447 per cent NaCl. The range of variation was 0.9318 to 0.9622. In women (16 subjects) the values varied between 0.9065 and 0.9401, the average being 0.9269. This difference between men and women has been related to the somewhat lower concentration in women of certain of the blood constituents, viz., urea, uric acid, bicarbonate, hemoglobin.

Muscle tissue is probably in osmotic equilibrium with the blood. During exercise, largely as a result of the increased formation of various metabolites, the osmotic pressure of the blood is elevated. This has been clearly demonstrated by the work of Margaria. A runner, 1½ min. after a steeplechase of 2 miles, which was covered in 11 min. 15 sec., had a specimen of blood taken. The osmotic pressure was found to be equivalent to 1.0482 ± .0026 per cent NaCl, an increase of about 10 per cent above the normal value. In another subject, after a standing run of 1½ min., the osmotic pressure of blood was equivalent to 1.033 per cent NaCl.

The osmotic pressure of the blood is lowered by water drinking. In one subject, Margaria observed it to diminish from an initial value of 0.9341 to a minimum of 0.8819, attained 1½ hours after drinking 1500 cc. of tap water.

Electrolytic Dissociation. The degree of dissociation of electrolytes in solution is measured by determining the electrical conductivity of the solution. For description of methods, the student is referred to modern textbooks on physical chemistry. Acids, bases, and salts are electrolytes and in water dissociate into *cations* (positively charged ions) and *anions* (negatively charged ions).[23]

Dissociation of Acids. An acid, HA, will dissociate as follows:

$$HA \rightleftarrows H^+ + A^-$$

Applying the mass-law equation,

$$\frac{[H^+] \times [A^-]}{[HA]} = K_a$$

[21] The student is referred to A. V. Hill, "Adventures in Biophysics," Lecture III, "The Conception of the Steady State," and also to the following articles: R. Margaria, "The Osmotic Changes in Some Marine Animals," *Proc. Roy. Soc.* (*London*), *B*, 107, 606 (1931); M. Duval, *Ann. Inst. oceanogr. Monaco*, N. S., 2, 233 (1925); C. Schlieper, *Z. vergl. Physiol.*, 9, 478 (1929); C. F. A. Pantin, *J. Exptl. Biol.*, 8, 63, 73, 82 (1931).

[22] *J. Physiol.*, 70, 417 (1930).

[23] For a fuller discussion consult D. I. Hitchcock, "Physical Chemistry for Students of Biology and Medicine," 2d edition, 1934.

K_a is the dissociation constant of the acid in question; $[H^+]$, the concentration of hydrogen ions; $[A^-]$, the concentration of the negative ions, and [HA], the concentration of the undissociated acid.

Dibasic acids dissociate in two steps; for each step there is a different constant, as follows:

$$H_2A \rightleftarrows H^+ + HA^-$$

$$HA^- \rightleftarrows H^+A^-$$

Applying the mass law to this acid,

$$\frac{[H^+] \times [HA^-]}{[H_2A]} = K_1; \quad \frac{[H^+] \times [A^-]}{[HA^-]} = K_2$$

For oxalic acid, $K_1 = 3.8 \times 10^{-2}$, $K_2 = 4.9 \times 10^{-5}$; for tartaric acid, $K_1 = 9.7 \times 10^{-4}$, $K_2 = 4.5 \times 10^{-5}$.

Phosphoric acid is tribasic and ionizes in three steps:

$$H_3PO_4 \rightleftarrows H^+ + H_2PO_4^-$$
$$\updownarrow$$
$$H^+ + HPO_4^=$$
$$\updownarrow$$
$$H^+ + PO_4^{\equiv}$$

The three constants are:

$$K_1 = 9 \times 10^{-3}; \quad K_2 = 8.8 \times 10^{-8}; \quad K_3 = 3.6 \times 10^{-13}.$$

Acids that are highly dissociated (strong acids) will give, according to the mass-law equation, a high value for the dissociation constant K. Weakly dissociated acids will give low values. Acid solutions of equivalent normality have the same amount of replaceable hydrogen, but the concentration of hydrogen ions in an acid solution depends on the degree of dissociation of the acid. Thus, 0.1 N hydrochloric acid contains the same amount of replaceable hydrogen as 0.1 N acetic acid, but the concentration of hydrogen ions in the former is approximately 70 times greater than in the latter, inasmuch as the hydrochloric acid (0.1 N) is 92 per cent dissociated and the acetic acid (0.1 N) only 1.3 per cent. Physiologically, the effect of acids frequently depends on the concentration of hydrogen ions.

Dissociation of Bases. Bases ionize according to the equation:

$$BOH \rightleftarrows B^+ + OH^-$$

where B is any basic radical. The dissociation constant is derived from the relation:

$$\frac{[B^+] \times [OH^-]}{[BOH]} = K_b$$

Ionization of Water. In view of the preceding discussion it is nat-

ural to inquire regarding the behavior of water. Water dissociates according to the equation:

$$HOH \rightleftarrows H^+ + OH^-$$

From the mass law, it follows that

$$\frac{[H^+] \times [OH^-]}{[H_2O]} = K$$

However, the degree of dissociation is so slight that the concentration of the undissociated portion is very nearly the same as the total concentration. Accordingly, it is permissible to simplify the equation to

$$[H] \times [OH] = K_W$$

The dissociation constant for water has been measured by many investigators, the value generally accepted being $K_W = 1.012 \times 10^{-14}$ at 25° C. Since on dissociation a molecule of water yields one hydrogen ion and one hydroxyl ion,

$$[H] = [OH] = \sqrt{K_W} = 1.006 \times 10^{-7}$$

or approximately 1×10^{-7}. This means that 1 liter of water contains one ten-millionth of a gram of hydrogen ions, or that there is 1 gram of hydrogen ions in 10 million liters of water. A tenth-normal solution of hydrochloric acid contains 1 gram of hydrogen ions in 10 liters of water.

***p*H.** For several reasons it is usually more convenient to express hydrogen-ion concentration in simplified form. In 1909, Sørensen pointed out that there were advantages in designating hydrogen-ion concentration in terms of the logarithm (to the base 10) of its reciprocal. This suggestion has since been accepted universally. Sørensen gave to $\log \frac{1}{[H]}$ the symbol P_H, but for typographical reasons it has been found more convenient to use the form *p*H. Thus, a neutral solution has a hydrogen-ion concentration (C_H) or $[H^+]$ value of 1×10^{-7}. The *p*H value of such a solution is therefore $\log \frac{1}{1 \times 10^{-7}}$, or $\log 10^7$, or 7.

If the hydrogen-ion concentration is given, the corresponding *p*H may be calculated as follows:

$$[H^+] = 2 \times 10^{-6}$$

$$pH = \log \frac{1}{[H]}; \quad \therefore \; pH = \log \frac{1}{2 \times 10^{-6}}$$

$$= \log 1 - \log (2 \times 10^{-6})$$

Since

$$\log 1 = 0, \; p\text{H} = -\log (2 \times 10^{-6})$$
$$= -\log 2 - \log 10^{-6}$$
$$= -0.301 + 6$$
$$p\text{H} = 5.699, \text{ or approximately } 5.7.$$

*p*H values may be converted into hydrogen-ion concentration as follows:

$$p\text{H} = 2.3$$
$$= 3 - .7$$
$$= 3 - \log 5.01$$
$$= \log \frac{1}{5.01 \times 10^{-3}}$$
$$[\text{H}^+] = 5.01 \times 10^{-3}.$$

Acid solutions have a *p*H range below 7, whereas the range of alkaline solutions is above 7. The following table of figures, showing approximately the relation of normality of HCl and NaOH solutions to *p*H, will make this point clear. For this purpose the assumption is made that the acid and alkali are completely ionized.

TABLE V

Normality	Concentration of H Ions	Concentration of OH ions	*p*H	*p*OH
N HCl	1	10^{-14}	0	14
0.1HCl	10^{-1}	10^{-13}	1	13
0.01HCl	10^{-2}	10^{-12}	2	12
0 001HCl	10^{-3}	10^{-11}	3	11
0.0001HCl	10^{-4}	10^{-10}	4	10
0.00001HCl	10^{-5}	10^{-9}	5	9
0.000001HCl	10^{-6}	10^{-8}	6	8
0.0000001HCl	10^{-7}	10^{-7}	7	7
Neutrality	10^{-7}	10^{-7}	7	7
0.0000001NaOH	10^{-7}	10^{-7}	7	7
0.000001NaOH	10^{-8}	10^{-6}	8	6
0.00001NaOH	10^{-9}	10^{-5}	9	5
0.0001NaOH	10^{-10}	10^{-4}	10	4
0.001NaOH	10^{-11}	10^{-3}	11	3
0.01NaOH	10^{-12}	10^{-2}	12	2
0.1NaOH	10^{-13}	10^{-1}	13	1
N NaOH	10^{-14}	1	14	0

As stated, these values are approximate, since neither HCl nor NaOH is 100 per cent ionized.

The Determination of Hydrogen Ions. In the present volume it is possible only to refer to the two methods that are in common use for

the determination of hydrogen ions. The first is an electrometric method; the second involves the use of indicators. Valuable treatises on this subject have been prepared by W. M. Clark,[24] Michaelis,[25] Kolthoff and Furman, and Kolthoff.[26]

Of the two methods, the one based on the use of indicators lends itself more readily to a brief description. Organic indicators are dyes which are essentially weak acids or weak bases and give rise to color changes in varying degrees of acidity and alkalinity. A familar example of an acid indicator is phenolphthalein. This compound exists in two tautomeric forms; in acid solution it is in the colorless form, whereas when added to an alkaline solution it acquires a magenta color. Methyl orange is a weak base which in acid solution is pink and in basic solution yellow. A number of indicators exhibit gradations in color within certain ranges of hydrogen-ion concentration and have therefore found extensive application in biochemistry.

The color that develops when a given indicator is added to a solution of unknown *p*H may be compared with the color given by the same indicator in solutions of known hydrogen-ion concentration. These standard solutions are usually prepared from mixtures of highly purified electrolytes which act as buffers, a buffer solution [27] being one that does not readily change its hydrogen-ion concentration upon the addition of small amounts of acid or alkali. The buffer solutions used frequently as standards contain the following constituents.

TABLE VI

Constituents	For *p*H Range
KCl--HCl	1 2- 2 2
KH Phthalate—HCl	2 2- 3 8
KH Phthalate—NaOH	4 0- 6 2
KH_2PO_4—NaOH	5 8- 8 0
KCl—NaOH	7 8-10 0

[24] W. M. Clark, "The Determination of Hydrogen Ions," 3d edition, Williams & Wilkins, Baltimore, 1928.

[25] L. Michaelis, "Hydrogen Ion Concentration," translated by W. A. Perlzweig, Williams & Wilkins, Baltimore, 1926.

[26] I. M. Kolthoff and N. H. Furman, "Potentiometric Titrations," John Wiley & Sons, New York, 1926; I. M. Kolthoff, "The Colorimetric and Potentiometric Determination of *p*H," John Wiley & Sons, New York, 1931.

[27] A buffer solution is a mixture of a weak acid and its alkali salt, or a weak base and its acid salt, as for example acetic acid and sodium acetate, potassium acid phosphate and dibasic phosphate. If to a mixture such as acetic acid and sodium acetate, acid (HCl) is added, some of the sodium acetate is converted into acetic acid, which is very weakly ionized. The addition of base, on the other hand, would result in the formation of more acetate. In either event the change in hydrogen-ion concentration would be insignificant compared to the change that would occur if there were no buffer. The stability of the solution, i.e., its resistance to change in reaction, is due to its *buffer action.*

Buffer standards may be prepared for a wide range of *p*H values. A large number of dyes are now also available for the determination of hydrogen-ion concentration. Among these are the following indicators:

TABLE VII

Indicator	*p*H Range	Indicator	*p*H Range
Thymol blue	1.2–2.8	Bromthymol blue	6.0–7.6
Bromphenol blue	3.0–4.6	Phenol red	6.6–8.2
Methyl red	4.4–6.0	Cresol red	7.2–8.8
Bromcresol purple	5.4–7.0	Thymol blue	8.2–9.8

As we proceed from one phase of biochemistry to another, we shall see the important bearing of hydrogen ions upon physiological phenomena.

The Colloidal State of Matter. The student is no doubt familiar with the observations of Thomas Graham,[28] who, in 1861, found that certain substances—urea, sodium chloride, sucrose, etc.—readily diffused through parchment membranes, but that other substances—gelatin, egg albumin, starch, etc.—failed to do so. To distinguish the two classes of substances, Graham called the first group crystalloids, and the non-diffusible substances, because of their glue-like character, colloids. Aqueous colloidal solutions Graham called hydrosols. When sufficiently concentrated, these set to a gel, hence the name hydrogel.

A substance may, however, under one set of conditions, exhibit the properties of a crystalloid, but, under different conditions, behave as a colloid. For example, sodium chloride dissolved in water is a crystalloid; in benzene it forms a colloidal solution. It is more correct, therefore, to speak of a substance as being either in the colloid or the crystalloid state.

Colloids are usually, though not always, amorphous, and in water frequently form viscous solutions. However, the real criterion of the colloidal state is that the particles are so much larger than molecules that they possess surface, and yet are not so large as to settle out easily by the action of gravity. In true solution, a substance exists either as ions, molecules, or small aggregates of molecules. The individual particles cannot be distinguished even with the aid of the ultramicroscope. Solutions of this type are said to be *homogeneous*; there is but one "phase," a term first employed in this sense by the distinguished American scientist, J. Willard Gibbs. Colloidal systems, on the other hand, are made up of more than one phase, and are therefore *heterogeneous*. Suppose that a solid mass, such as a bar of gold, is placed in water. This would constitute a two-phase system, the solid mass being one

[28] *Liebig's Ann. Chem.*, **121**, 1 (1862).

phase; the water, the other. There would still be two phases even though the gold bar were subdivided into smaller and smaller particles until particles of colloidal dimension were obtained. In a colloidal system, the suspended particles constitute the *dispersed* or *internal phase* (also dispersoid); the other phase is the *dispersion medium* or *external phase* (also continuous phase).

Surface. One of the characteristics of a dispersed system is the development of the surface of contact between the phases. A 1-cm. cube has a surface of 6 sq. cm. Subdivision of this into exceedingly small cubes, having 0.1 micron as the side dimension, would result in the development of the surface to 60 sq. m. The question of surface is of much importance in physiological phenomena in which surface forces play a part, as in enzyme action. Many substances tend to become concentrated at a surface, this phenomenon of surface condensation being known as *adsorption.* Adsorption is an important property of colloids for the reason that these have a large surface as compared with their mass. Adsorption is thought to be a factor in the staining reactions of tissues, the action of drugs, and many other phenomena.

Suspensoids and Emulsoids. The division of colloids into two classes is based on the ability of some colloids to take up water. Kaolin, platinum, gold, arsenious sulfide, etc., when in the colloidal state consist of pure solid and are hence classified as *suspensoids* (also lyophobic, or hydrophobic, literally "water-hating," colloids). Other colloids in aqueous solution have so marked an attraction for water that the dispersoid may contain large quantities of water. These are the *emulsoids* (also hydrophilic or lyophilic, literally "water-loving," colloids). The latter group is of primary importance physiologically, protoplasm being essentially an emulsoid type of colloid. Other familiar examples are starch, soap, egg white, and gelatin. No confusion should arise from the fact that colloidal systems in which both phases are liquid are usually classified as emulsions. The name emulsoid is properly confined to those cases in which the internal phase contains more or less water, although it may sometimes more closely approximate a solid. It would be impossible to give an acceptable classification of colloids without bringing into the discussion a considerable amount of detail and the conflicting points of view of several authorities. This information belongs more properly to works devoted to colloid chemistry.[29]

Viscosity. Viscosity is due to the internal friction of the molecules of a liquid. Solutions are almost always more viscous than the pure solvents. The viscosity of suspensoids in water is not much greater

[29] For a comprehensive survey of the theoretical aspects of colloid chemistry, as well as of its applications in biology and medicine, the student is referred to "Colloid Chemistry—Theoretical and Applied," edited by J. Alexander, Chemical Catalog Co., New York, Vol. I (1926); Vol. II (1928); Vol. III (1930); Vol. IV (1932).

than that of the water, but in emulsoids the viscosity is very markedly increased. Thus, the viscosity of 1 per cent agar is several thousand times that of water. Increasing the concentration of the dispersed phase, particularly in emulsoids, increases the viscosity of colloidal systems.

Size of Colloidal Particles. The limit of vision with the aid of the microscope is about 0.0001 mm. or 0.1 μ.[30] Colloidal particles range in diameter between 1 mμ and 100 mμ (1 μ = 0.001 mm.; 1 mμ = 0.001 μ) and therefore are below the range of microscopic visibility. It is possible, however, to detect particles of colloidal dimension by their diffraction images. Faraday, and later Tyndall, observed that, when a beam of light is sent through a clear solution of finely divided gold, some of the light is diffracted by the solid particles. The effect is the same when a beam of sunlight passes into a darkened room through a small opening. The light is made visible because of the scattering of a portion of it by the particles of dust in its path. In turn, the motion of these particles is made visible in the diffused light. Even distilled water may contain particles which exhibit a similar effect, but the phenomenon is much more apparent in colloidal suspensions. This is known as the Tyndall or Faraday-Tyndall phenomenon and is of importance because upon this principle is based the use of the "dark field" or ultramicroscope.

Electric Charge. Colloidal particles usually carry either a positive or a negative electric charge and are attracted to poles of opposite sign. The neutralization of the charge of colloidal particles causes them to be precipitated. The movement of electrically charged particles toward an oppositely charged electrode is known as *cataphoresis*. Hemoglobin, ferric hydroxide, and aluminum hydroxide are electropositive; gold, silver, platinum, arsenious sulfide, kaolin, and charcoal are examples of electronegative colloids.

Donnan's Theory of Membrane Equilibria. The application of Donnan's theory[31] of membrane equilibria to physiological problems has received much attention during the past few years. Briefly stated, the theory defines the relations which exist between the ions of a solution of electrolytes separated by a membrane which is impermeable to one of the ions. Let us consider, as an illustration, two electrolytes, NaR and NaCl, on opposite sides of a membrane represented below by a vertical line.

(1)	(2)
Na^+	Na^+
R^-	Cl^-

[30] μ = micron; mμ = millimicron.

[31] Donnan, *Chem. Rev.* 1, 73 (1924). For a later discussion consult the monograph of T. L. Bolam, "The Donnan Equilibria," G. Bell & Sons, Ltd., London, 1932.

Of these ions R^- cannot diffuse through the membrane. At equilibrium therefore, the following condition will exist.

(1)	(2)
Na^+	Na^+
R^-	
Cl^-	Cl^-

From the principles of thermodynamics it has been deduced that *at equilibrium the products of the concentrations of the diffusible ions on each side of the membrane are equal.* Remembering that on one side of the membrane there is a non-diffusible ion, R^-, the very fact that the product of the concentrations of the diffusible Na^+ and Cl^- ions on one side of the membrane is equal to their product on the other shows that the concentration of either ion on one side is different from its concentration on the other. On the same side containing the non-diffusible ion R^-, the concentration of the cation Na^+ is the sum of the cations combined with the non-diffusible anion, R^-, plus the cations in combination with Cl^-. But on the other side of the membrane the concentration of the Na^+ ions is only that of Na^+ combined with Cl^- and equal to the concentration of Cl^-. Therefore to fulfill Donnan's equation which is

$$[Na^+]_1 \times [Cl^-]_1 = [Na^+]_2 \times [Cl^-]_2$$

the following conditions must exist:

$$[Na]_1 > [Na]_2$$

and

$$[Cl]_1 < [Cl]_2$$

The above is a simple illustration of Donnan's theory. The situation is more complex in dealing with a large number of ions.

As we shall see, this inequality of distribution of ions on the opposite sides of a membrane is frequently encountered in biological systems.

CHAPTER II

THE CARBOHYDRATES [1]

Concerning the mode of formation of carbohydrates in the plant, there is much difference of opinion, although it has been recognized for many years that chlorophyll, the green coloring matter of plants, is in some way involved in the synthesis and that the carbon is derived from carbon dioxide. The theory which first gained wide acceptance was proposed by Baeyer [2] in 1870. According to Baeyer's hypothesis, the first step in carbohydrate synthesis in the plant is a reduction of carbon dioxide to carbon monoxide, which is then further reduced to formaldehyde. This is followed, according to the theory, by condensation of the formaldehyde, yielding sugars and polysaccharides.

The conception that formaldehyde is the first stage in the photosynthetic process in the green leaf is based partly on indirect experimental evidence. Butlerow [3] treated trioxymethylene, a polymer of formaldehyde, with lime and obtained a sweetish syrup. Loew [4] later demonstrated the conversion of formaldehyde, *in vitro*, in the presence of bases, into a mixture of sugars. From this mixture, a fermentable sugar was isolated. Baly [5] and his students have described the reduction by ultraviolet light of carbon dioxide into formaldehyde and the condensation of the latter into reducing sugars. Other investigators, however, have been unable to confirm Baly's results.

It is considered that the primary process in photosynthesis consists in the absorption of light energy by chlorophyll, a property which endows this pigment with unique importance in nature. Chlorophyll exists in two forms, designated chlorophyll a and chlorophyll b, in which the following formulas were assigned by Willstätter and Stoll:[6]

Chlorophyll a $C_{55}H_{72}O_5N_4Mg$

Chlorophyll b $C_{55}H_{70}O_6N_4Mg$

[1] The International Union of Pure and Applied Chemistry has proposed the term "glucides" to embrace the carbohydrates and glucosides.

[2] *Ber.*, **3**, 63 (1870).

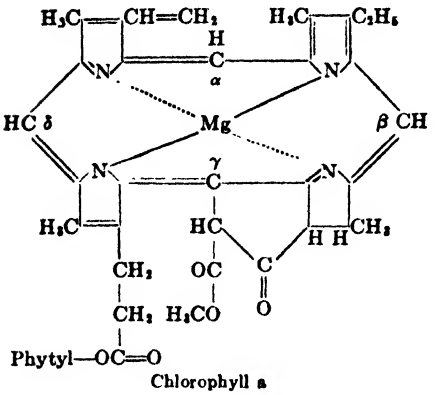
[3] *Ann. Chem. Pharm.*, **120**, 295 (1861).

[4] *J. prakt. Chem.*, N. F., **33**, 321 (1886); *Ber.*, **22**, 482 (1889).

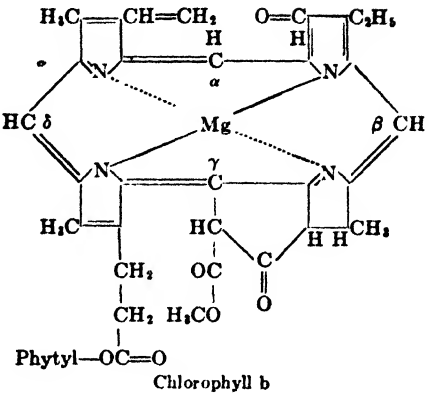
[5] *J. Chem. Soc.*, **119**, 1025 (1921); **121**, 1078 (1922); *Proc. Roy. Soc.* (*London*), *A*, **116**, 197, 212 (1927); *Science*, **68**, 364 (1928).

[6] R. Willstätter and A. Stoll, "Untersuchungen über die Assimilation der Kohlensaure," Berlin, 1918.

The following structural formulas have been proposed.[7]



Chlorophyll a



Chlorophyll b

Willstätter and Stoll developed the concept that chlorophyll combines with carbonic acid and that by a series of changes the latter is reduced to formaldehyde, in which form it is liberated from its union with the chlorophyll.

Many other explanations of the photosynthesis of formaldehyde have been advanced. Franck,[8] for example, assumes the photo-oxidation of chlorophyll during a preliminary induction period, subsequent to which the partly reduced chlorophyll participates in a series of four reactions, each involving the absorption of a quantum of energy. In these reactions H atoms and OH radicals are exchanged between chlorophyll, water, and carbon dioxide, the last being eventually oxidized to formaldehyde.

Van Niel[9] believes that the primary action of light may be the activation of hydrogen, one quantum of energy being required for each atom. Transfer of the activated hydrogen, which according to van Niel is derived from H_2O molecules, to carbon dioxide should result in the production of formaldehyde. If this hypothesis is correct, the oxygen liberated in the reaction is likewise derived from H_2O molecules. It is of interest that photosynthesis occurs in certain bacteria containing a pigment closely resembling chlorophyll and that there exist hydrogen donators other than water.

Chlorophyll readily loses two atoms of hydrogen by dehydrogenation, yielding dehydrochlorophyll. This fact is considered to be very

[7] H. Fischer and S. Breitner, *Ann.*, **522**, 151 (1936); Fischer and K. Bauer, *ibid.*, **523**, 235 (1936). See also the reviews on chlorophyll by C. C. Steele, *Chem. Rev.*, **20**, 1 (1937), and by H. Fischer, *ibid.*, p. 41.

[8] Details will be found in a brief review by J. Franck, *Chem. Rev.*, **17**, 433 (1935).

[9] C. B. van Niel, *Cold Spring Harbor Symposia on Quantitative Biology*, **3**, 138 (1935). The reader is referred to this volume for a series of excellent articles on various aspects of the subject of photosynthesis.

significant by Conant [10] and associates, who have suggested that the hydrogen thus made available is utilized in the reduction of carbon dioxide. This is the first stage of the photosynthetic reaction; it occurs in the dark and apparently depends on the intervention of an enzyme. Designating chlorophyll by Ch and dehydrochlorophyll by Ch(—2H), the first stage may be represented thus:

(1) Dark reaction:

$$12Ch + 6CO_2 \xrightarrow{\text{enzyme}} 12Ch(-2H) + \underset{\text{(glucose)}}{C_6H_{12}O_6} + 6H_2O$$

In the second stage, radiant energy is required for the regeneration of the chlorophyll.

(2) Light reaction:

$$12CH(-2H) + \text{light} + 12H_2O \rightarrow 12Ch + 6O_2$$

The light reaction is not affected by temperature and proceeds at a much greater speed than the dark reaction, which is dependent on temperature, being slower at low than at high temperatures.[11]

The exact nature of the first sugar formed in photosynthesis is not known definitely, but most of the experimental evidence indicates that it is probably a hexose.[12]

In summary it may be stated that the synthesis of carbohydrate in the plant depends on the absorption of light energy by chlorophyll a. It is not known to what extent other pigments occurring in the chloroplasts (chlorophyll b, carotene, and xanthophyll) participate in the photosynthetic process. The intermediate formation of formaldehyde from carbon dioxide is generally assumed, and although there is much evidence in support of this hypothesis, definite proof is lacking. Essentially, the process of photosynthesis consists in the transformation of radiant energy into chemical energy, which is stored in the plant in the form of carbohydrate, protein, and fat. This stored energy is the basis for practically all plant and animal life.

CLASSIFICATION OF THE CARBOHYDRATES

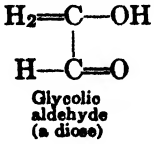
Glycolic aldehyde, containing one aldehyde and one primary alcohol group ($CH_2OH \cdot CHO$), is the simplest aldehyde having the properties commonly associated with the sugars. It is sweet to the taste, crystal-

[10] J. B. Conant, J. F. Hyde, W. W. Moyer, and E. M. Dietz, *J. Am. Chem. Soc.*, **53**, 359 (1931); Conant, Dietz, and S. E. Kamerling, *Science*, **73**, 268 (1931). Compare with R. B. Gordon, *Biol. Abstracts*, **6**, 683 (1932); cited by Spoehr, *Ann. Rev. Biochem.*, **2**, 453 (1933).

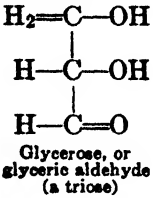
[11] R. Emerson and W. Arnold, *J. Gen. Physiol.*, **15**, 391 (1932); **16**, 191 (1932); see also Emerson, *ibid.*, **12**, 609 (1929); Arnold, *ibid.*, **17**, 135, 145 (1933).

[12] E. C. Barton-Wright and M. C. Pratt, *Biochem. J.*, **24**, 1217 (1930); H. F. Clements, *Plant Physiol.*, **7**, 547 (1932).

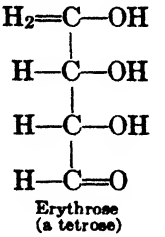
line, and readily soluble in water. The following compounds are usually grouped with the simple carbohydrates:



Glycolic aldehyde (a diose)



Glycerose, or glyceric aldehyde (a triose)



Erythrose (a tetrose)

Sugars having an aldehyde group are termed aldoses; those with a ketone (C=O) group are classified as ketoses. Of the latter, the lowest member is dioxyacetone ($CH_2OH \cdot CO \cdot CH_2OH$).

The Monosaccharides [13]

$(C_m(H_2O)_m)$

Except for the methyl pentoses, the general formula $C_m(H_2O)_m$ may be applied to the *simple sugars, or monosaccharides.* These may be classified as follows:

Biose (diose)—($C_2H_4O_2$)—glycolaldehyde (glycolose).
Trioses ($C_3H_6O_3$) —Aldose—glycerose.
Ketose—dioxyacetone.
Tetroses ($C_4H_8O_4$) —Aldoses—erythrose, threose.
Ketose—erythrulose.
Pentoses ($C_5H_{12}O_5$) —Aldoses—arabinose, xylose, lyxose, ribose.
Ketose—araboketose, xyloketose.
[Methyl pentoses ($C_6H_{10}O_5$)—rhamnose, fucose.]
Hexoses ($C_6H_{12}O_6$) —Aldoses—glucose, galactose, mannose, gulose, idose, talose, allose, etc.
Ketoses—fructose, sorbose, tagatose.
Heptoses ($C_7H_{14}O_7$) —Aldoses—mannoheptose, etc.
Ketoses—sedoheptose, etc.

Relatively few of the monosaccharides occur free in nature. Those found naturally are fructose, glucose, sedoheptose, and mannoheptose. Arabinose, xylose, galactose, mannose, and other monosaccharides may be obtained by fermentation or hydrolysis of naturally occurring substances. Others are merely laboratory products. Among these may be mentioned erythrose, lyxose, and gulose. Three octoses ($C_8H_{16}O_8$),

[13] The classification given here is based on that of Sherman in "Chemistry of Foods and Nutrition," Macmillan, 1928 edition, p. 10.

two nonoses ($C_9H_{18}O_9$), and one decose ($C_{10}H_{20}O_{10}$) have been prepared in the laboratory.

The Disaccharides

$(C_{12}H_{22}O_{11})$

The monosaccharides cannot be hydrolyzed into simpler carbohydrates, thus differing from the disaccharides and polysaccharides which on hydrolysis yield monosaccharides. For example,

$$C_{12}H_{22}O_{11} + H_2O \rightleftarrows 2C_6H_{12}O_6$$

The disaccharides may therefore be looked upon as being made up of two molecules of the same or of different monosaccharides from which one molecule of water has been abstracted. The general formula for these compounds would therefore be $C_{2m}(H_2O)_{2m-1}$.

1. Anhydrides of fructose and glucose: the best-known example is sucrose.
2. Anhydrides of glucose and galactose: e.g., lactose.
3. Anhydrides of glucose and glucose: maltose, isomaltose, trehalose, etc.

A variety of other disaccharides has been described. These include a dipentose saccharide, diarabinose, $C_{10}H_{18}O_9$, and several pentose-hexose saccharides.

The Trisaccharides

$(C_{18}H_{32}O_{16})$

By the elimination of two molecules of water from three monosaccharide molecules, we obtain the empirical formula for the trisaccharides $[C_{3m}(H_2O)_{3m-2}]$, of which the following are the most familiar compounds:

1. Anhydride of fructose + glucose + galactose: raffinose. Raffinose occurs in the sugar beet, cottonseed meal, etc.
2. Anhydride of glucose + fructose + glucose: melicitose (melezitose). This is found in the twigs of the larch and of the Douglas fir, and elsewhere.

The tetrasaccharides may be regarded as resulting from the condensation of four monosacoharide molecules with the loss of three molecules of water, $C_{4m}(H_2O)_{4m-3}$. The best-known example is stachyose ($C_{24}H_{42}O_{21}$), which on hydrolysis yields two molecules of galactose and one molecule each of fructose and glucose. It occurs in peas, ash manna, and the twigs of the white jasmine.

The Polysaccharides

The polysaccharides yield on complete hydrolysis either pentoses or hexoses and are obviously the polymerides of these molecular units.

I. Pentosans

$(C_5H_8O_4)_x$

The pentosans are the chief constituents of gums and mucilages.

(*a*) Xylans (anhydrides of xylose). These are found in straw, oat hulls, corn cobs, and most woods.

(*b*) Arabans (anhydrides of arabinose). The arabans are found chiefly in cherry gum or gum arabic.

II. Hexosans

$(C_6H_{10}O_5)_x$

(*a*) Glucosans (anhydrides of glucose). These are the most abundant of polysaccharides. Examples: cellulose, starch, dextrin, glycogen.

(*b*) Mannans or mannosans (anhydrides of mannose). These are found in the ivory nut, from which buttons are made, and in various legumes.

(*c*) Galactans (anhydrides of galactose). Many gums, agar, algae, lichens, and mosses contain galactans. Fruit pectins likewise contain galactans. A polysaccharide has been recently described which is analogous to glycogen, but yields galactose on hydrolysis; hence the name galactogen. It occurs, together with glycogen, in snails and exclusively in the eggs of the snail.

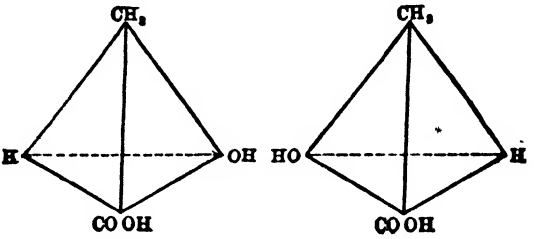
(*d*) Fructosans (anhydrides of fructose). Inulin, present in the tubers of the Jerusalem artichoke, is the most familiar example. It is also present in the bulbs of the onion and garlic. On hydrolysis, inulin yields fructose.

Optical Activity. A beam of light may be *polarized*, i.e., caused to vibrate in one plane, by passing through a Nicol prism. This consists of a specially constructed rhomb of Iceland spar or calcite (pure $CaCO_3$). When a beam of polarized light is passed through certain substances in solution, its plane may be turned either to the right or to the left. Such substances are said to be optically active; those that turn the plane of polarized light to the right are *dextrorotatory*, whereas those that turn the beam to the left are *levorotatory*. The polariscope is an instrument for measuring the angle through which the plane of polarized light is turned.

Pasteur [14] was among the first to appreciate that a relation existed between optical activity, chemical constitution, and biochemical behavior. In his epoch-making investigations on tartaric acid, he discovered that the so-called racemic acid, which was itself optically inactive, could be separated into two crystalline forms, one being the mirror image of the other. When separated, one form was dextrorotatory and the other levorotatory.

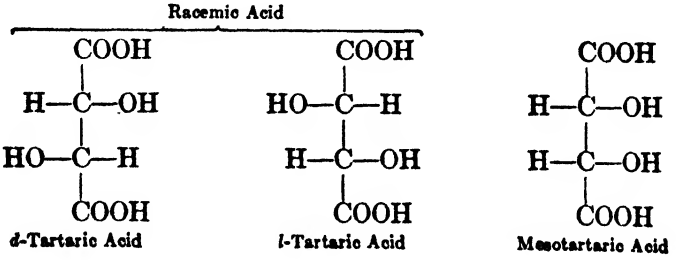
[14] *Ann. chim. phys.*, **24**, 442 (1838); **28**, 56 (1850); **31**, 67 (1851).

The problem was further elucidated by the work of Le Bel [15] and van't Hoff,[16] who independently established the relationship between optical activity in organic compounds and the presence in them of asymmetric carbon atoms. An asymmetric carbon atom is one united to four different atoms or groups of atoms. Lactic acid contains one asymmetric carbon atom. The spatial arrangement of the atoms in lactic acid attached to the center carbon atom may be represented as follows:



Lactic acid, therefore, occurs in two optically active forms. Inactive or racemic, lactic acid is a mixture of the two active forms, in equal proportions. It is important to realize that in laboratory syntheses of compounds containing asymmetric carbon atoms the products are usually optically inactive, since by the law of probabilities an equal number of molecules of the levo- and dextrorotatory forms would be obtained. Natural syntheses result in the formation of substances which may and frequently do show optical activity.

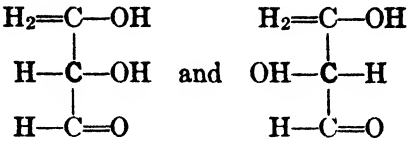
Tartaric acid contains two asymmetric carbon atoms and exists in four forms, namely as (*a*) inactive racemic acid which may be separated into (*b*) dextro-tartaric and (*c*) levo-tartaric acids; and (*d*) mesotartaric acid which is inactive because of "internal compensation" within the molecule. These compounds may be represented structurally as follows:



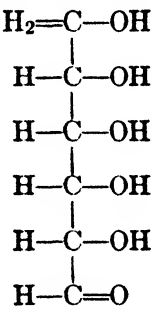
Stereoisomerism and the Constitution of the Monosaccharides. If the structural formula of glycerose is examined (p. 27), it will be

[15] *Bull. soc. chim.*, **22**, 337 (1874).
[16] *Ibid.*, **23**, 295 (1875).

seen that the middle carbon atom is asymmetric, since it is united to four different atoms or groups of atoms. Accordingly, there must exist a dextrorotatory and a levorotatory form of glycerose in addition to the inactive or racemic form, which is a mixture in equal proportions of the two optically active varieties. The optically active forms of glycerose may be represented as follows:



According to the Le Bel-van't Hoff hypothesis, if n represents the number of asymmetric carbon atoms in a given sugar molecule, then 2^n will be the total number of active forms of the sugar. Applying the rule to a 4-carbon-atom sugar having 2 asymmetric carbon atoms, we obtain 4 as the number of isomers. These are *d*- and *l*-erythrose and *d*- and *l*-threose. Similarly, 8 aldopentoses—*d*- and *l*-arabinose, *d*- and *l*-xylose, *d*- and *l*-ribose, and *d*- and *l*-lyxose—are theoretically possible. In the case of the aldohexose sugars, the empirical formula indicates the presence of 4 asymmetric carbon atoms.

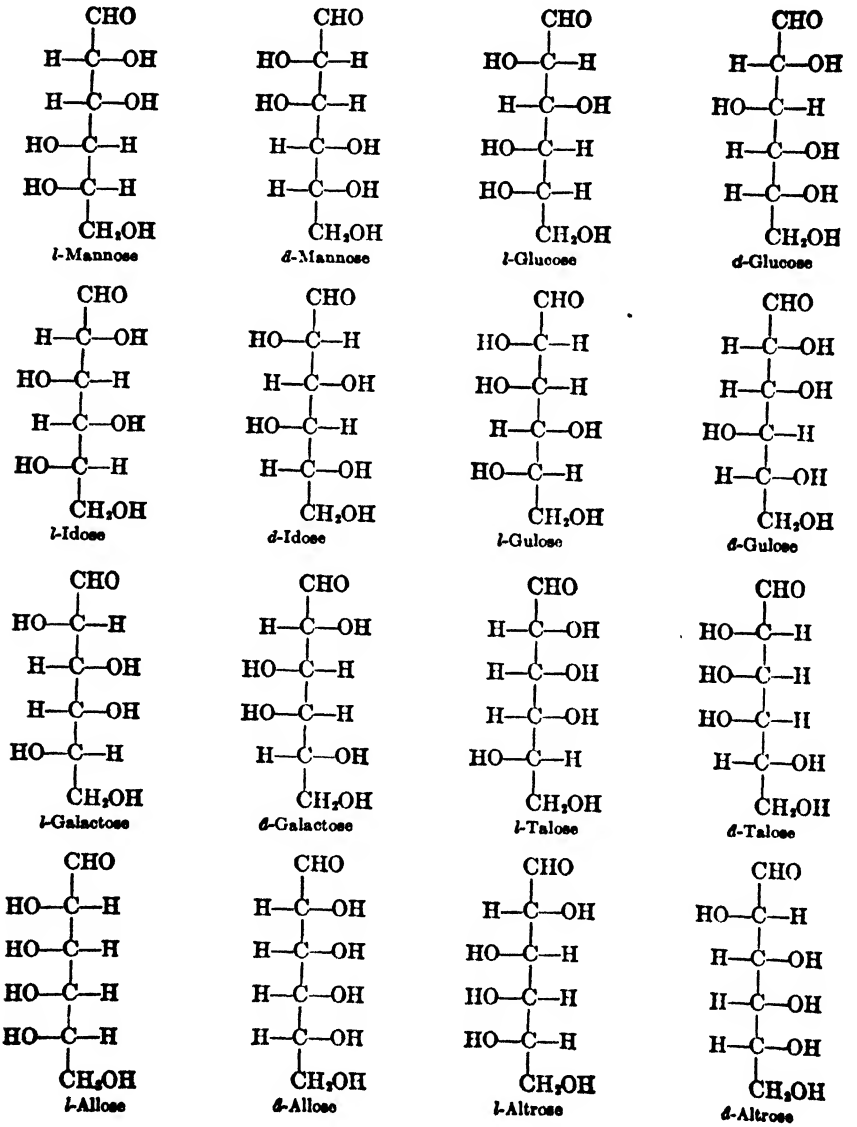


On this basis, 16 stereoisomeric modifications are theoretically possible. Except for *d*- and *l*-glucose, the designations *d*- and *l*- do not necessarily imply dextrorotation and levorotation. A *d*-sugar is a sugar which is structurally related, so far as the asymmetry of its carbon atoms is concerned, to *d*-glycerose. An *l*-sugar is similarly structurally related to *l*-glycerose. Of the 16 sugars, 3 are known to occur naturally either free or in combination (glucose, mannose, galactose), and the others have been prepared in the laboratory by Emil Fischer and his followers (p. 32).

The number of aldohexose isomerides would be limited to 16 on the assumption that the structure of the aldohexose molecule is that of an open-chain aldehyde. The structural formula that the organic chemist assigns to a given organic compound is determined by what is known concerning its physical and chemical properties. If, from this standpoint, the best known of the aldohexoses, namely *d*-glucose, is subjected

to a critical examination, it is discovered that the straight-chain aldehyde formula, as given above, does not fully account for all the chemical and physical properties of this compound.

THE ALDOHEXOSES



Reactions. On heating glucose with a concentrated solution of hydriodic acid, the oxygen is removed and it is converted to *n*-secondary-

hexyliodide, $[CH_3 \cdot CH_2 \cdot CHI \cdot CH_2 \cdot CH_2 \cdot CH_3]$, which is a derivative of *n*-hexane, $[CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_3]$. This proves that glucose is a straight-chain compound.

The hydroxyl groups react with metals to form compounds resembling the "alcoholates." When glucose is treated with acids, acid anhydrides, and acid chlorides, esters are formed in which 5 hydrogen atoms of the hydroxyl groups are replaced by acid radicals. An example of such an ester is glucose pentacetate $[C_6H_7O(O \cdot CO \cdot CH_3)_5]$. This is evidence that there are five hydroxyl groups, and, because of the stability of glucose, it is to be assumed that each hydroxyl group is associated with a different carbon atom.

The aldehyde group of glucose may be reduced with sodium amalgam to an alcohol group, yielding a hexahydric alcohol, sorbitol, $[CH_2OH(CHOH)_4CH_2OH]$, or it may be oxidized to give gluconic acid, $[COOH(CHOH)_4CH_2OH]$. Oxidation of the terminal primary alcohol group yields glycuronic acid, $[COOH(CHOH)_4CHO]$, then saccharic acid, $[COOH(CHOH)_4COOH]$, as the end-product. Because of the aldehyde group, glucose reduces alkaline copper solutions and other metallic hydroxides. It reacts with hydrocyanic acid to yield glucose cyanhydrin:

$$CH_2OH(CHOH)_4CHO + HCN = CH_2OH(CHOH)_4C(H)(OH)(CN)$$

This, on hydrolysis, gives glucoheptonic acid, which, on reduction, yields a sugar containing seven carbon atoms, namely, glucoheptose, $[CH_2OH(CHOH)_5CHO]$. Treated with hydroxylamine, glucose yields the corresponding oxime;[17] with phenylhydrazine, glucosazone is obtained.[18] Taken by themselves, these reactions would seem to afford

[17] $CHO \cdot (CHOH)_4 \cdot CH_2OH$ (Glucose) $+ H_2N \cdot OH$ (Hydroxylamine) $= CH:N \cdot OH \cdot (CHOH)_4 \cdot CH_2OH$ (Glucose-oxime) $+ H_2O$

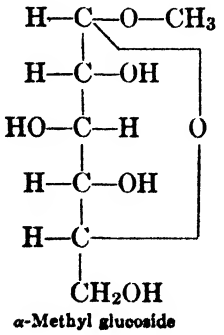
[18] $CHO \cdot CHOH \cdot (CHOH)_3 \cdot CH_2OH$ (Glucose) $+ C_6H_5 \cdot NH \cdot NH_2$ (Phenylhydrazine) $= CH:N \cdot NH \cdot C_6H_5 \cdot CHOH \cdot (CHOH)_3 \cdot CH_2OH$ (Phenylhydrazone of glucose) $+ H_2O$

$CH:N \cdot NH \cdot C_6H_5 \cdot CHOH \cdot (CHOH)_3 \cdot CH_2OH + 2C_6H_5NH \cdot NH_2 = C:N \cdot NH \cdot C_6H_5 \cdot (CHOH)_3 \cdot CH_2OH$ (Glucosazone) $+ C_6H_5NH_2 + NH_3 + H_2O$

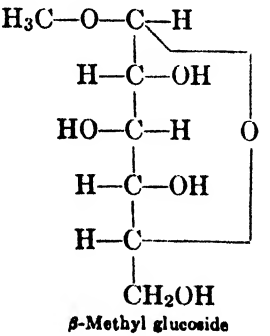
sufficient proof for the open-chain aldehydic structure of the glucose molecule; but when the reactivity of the compound is compared with that of other hydroxy aldehydes, it becomes apparent that glucose is not as reactive as might be expected from the simple aldehyde formula. Indeed, if the structure of glucose were as represented by the open-chain aldehyde formula, it would be impossible to explain certain of its physical properties.

Mutarotation. It has been observed that the optical rotation of a freshly prepared aqueous solution of glucose or of any other sugar containing a free aldehyde group changes on standing. The initial specific rotations of *d*-glucose prepared in different ways may be totally different. Thus glucose prepared by recrystallization from acetic acid gives, when freshly dissolved in water, a rotation $[\alpha]_D$ of about $+110°$ (see p. 55). Prepared by crystallization from an aqueous solution above 98° C., the *d*-glucose exhibits an initial rotation $[\alpha]_D$ of about $+19°$. Either solution, when allowed to stand, very slowly changes its rotation until a value of $[\alpha]_D$ of $+52.5°$ is obtained. The change occurs almost immediately if a small amount of alkali is added. This phenomenon, first observed by Dubrunfaut in 1846, is known as *mutarotation* (also birotation or multirotation) and is believed to be due to the conversion of one form of the sugar into another having a different molecular configuration and hence a different optical rotation.

Glucoside Formation. When glucose is treated with methyl alcohol in the presence of dry hydrochloric acid gas, two distinct compounds are formed, neither having an aldehyde group. Both compounds are methyl glucosides, and one may be separated from the other by fractional crystallization. The methyl glucosides do not behave as aldehydes. It has been determined that in each case a methyl group replaces a hydrogen atom belonging to an hydroxyl group attached to the carbon atom which is supposedly part of the aldehyde group of the glucose molecule. The two methyl glucosides have been distinguished by the prefixes α and β, and for reasons to be considered presently have been represented structurally by the following formulas:



α-Methyl glucoside



β-Methyl glucoside

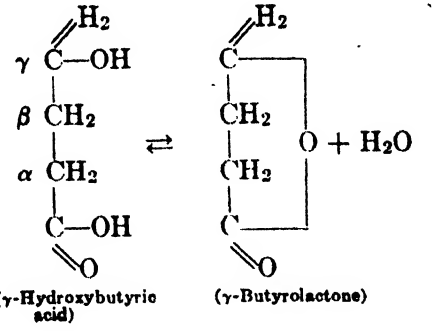
The two compounds differ in solubility, melting-point, rotatory power, and crystalline form, the α-glucoside crystallizing in long needles, and the β-form in rectangular prisms.

	Melting-point	Rotatory Power
α-Methyl glucoside	165°	+157°
β-Methyl glucoside	104°	- 33°

The methyl glucosides also differ in their reactions toward enzymes. Maltase acts only on the α glucoside, having no effect on the β-form. The latter is acted on by the enzyme emulsin. The pure β-form may be prepared by incubating the mixture of the two glucosides with ordinary baker's yeast. The maltase contained in the yeast hydrolyzes the α-methyl glucoside, and the zymase, which is also present, ferments the resulting glucose to carbon dioxide and ethyl alcohol. The β-methyl glucoside remains unaffected.

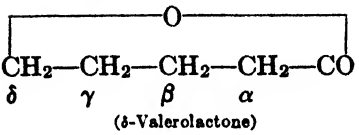
The behavior of ordinary glucose in forming two methyl glucosides suggests that an hydroxyl (OH) group is probably available at the terminal (aldehyde) carbon atom. It also indicates that two distinct forms of glucose are acted upon to yield the two methyl glucosides.

Lactones and Lactals. Before continuing the discussion of the molecular structure of glucose, it is permissible to digress briefly in order to consider certain cyclic compounds with which the reader is already familiar. It will be recalled that a distinguishing feature of γ- and δ-hydroxy acids is their ability to form cyclic esters, the carboxyl group entering into reaction with the hydroxyl group. The formation of these cyclic compounds is accelerated by mineral acids, whereas cleavage of the lactone linkage may be accomplished through the agency of alkali hydroxides and carbonates. As an illustration of a γ-lactone, we have:



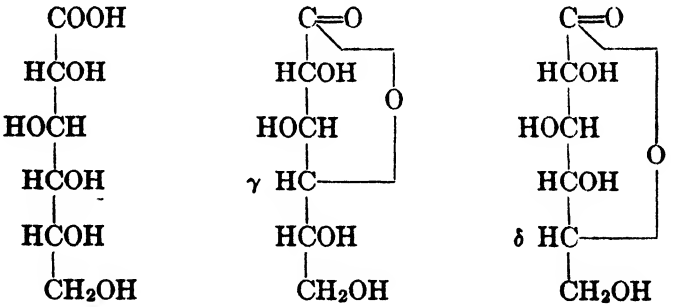
(γ-Hydroxybutyric acid) (γ-Butyrolactone)

The following is the formula of the lactone of δ-hydroxyvaleric acid:



(δ-Valerolactone)

The sugar acids, which may be formed by the oxidation of the aldehyde group to a carboxyl group, show the same tendency to form lactones. In a careful study of the relation between the chemical constitution and the optical rotatory power of 24 different sugar-acid lactones, Hudson [19] advanced the hypothesis, now usually referred to as Hudson's "lactone rule," that lactones which are dextrorotatory have the lactone ring on one side, represented on the right side of the structure, whereas lactones which are levorotatory have it on the other side, and that the position of the ring shows the former position of the OH group on the γ-carbon atom. Hudson concluded that the sugar acids form γ-lactones. Although this configuration is undoubtedly the predominant one, evidence has, however, accumulated [20] pointing to the existence of δ-lactones of sugar acids. Thus, gluconic acid is said to yield at least two lactones, namely a γ- and a δ-lactone:



The lactones of the sugar acids are relatively stable, and some have been prepared in crystalline form. Levene [21] is of the opinion that, in a solution of sugar acids, all theoretically possible lactones are formed. In a freshly prepared solution the unstable lactones are said to predominate, but after a short time only the stable forms are present in measurable quantities.

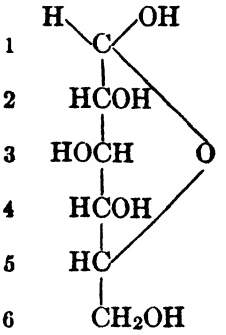
Analogous to the formation of lactones from the sugar acids are the intramolecular rearrangements which the sugars themselves exhibit. Here also, an oxygen bridge is introduced which may be supposed to link carbon atoms 1 and 4 (γ-linkage), 1 and 5 (δ-linkage), etc.

[19] *J. Am. Chem. Soc.*, **32**, 338 (1910).

[20] J. U. Nef, *Ann. Chem.*, **403**, 204 (1914); W. N. Haworth and V. S. Nicholson, *J. Chem. Soc.*, **129**, 1899 (1926).

[21] *Chem. Rev.*, **5**, 1 (1928).

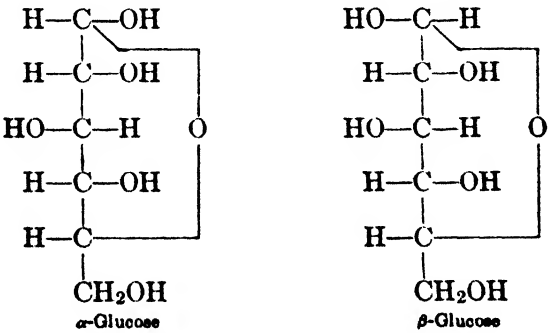
Assuming for the present the <1,5> linkage, the glucose molecule may be represented by the formula:



Levene refers to compounds of this type as "lactals," a term originally suggested by Helferich and Fries.[22]

Structure of the Glucose Molecule. We may now consider whether the lactal structure of the glucose molecule offers an adequate explanation for certain physical and chemical properties of glucose which we are unable to account for on the basis of the open-chain aldehyde structure.

It will be observed that in the lactal formula for glucose just given, in addition to the asymmetry of carbon atoms 2, 3, 4, and 5, the terminal carbon atom 1 is also asymmetric. Consequently, two isomeric modifications of *d*-glucose are possible, depending on the space relations of the terminal H and OH groups. In accordance with the nomenclature used in describing the methyl glucosides (p. 34), the formulas for glucose, referred to as the α- and β-amylene oxide forms, may be written:



The existence of two forms of *d*-glucose was demonstrated by Tanret[23] in 1896. He described an "α" glucose ($[\alpha]_D = +110°$) which on standing changed its specific optical rotation to the equilibrium

[22] *Ber.*, **58**, 1246 (1925).
[23] *Bull. soc. chim.*, **15**, 195, 349 (1896).

point ($[\alpha]_D = +52.5°$). Another sugar of rotation ($[\alpha]_D = +19°$) increased its rotatory power to 52.5°. These observations have been confirmed by other investigators and help to explain the phenomenon of mutarotation. Both the α- and the β-forms of glucose are present when ordinary anhydrous glucose is dissolved, and the initial optical rotation, in any given case, will depend on the relative proportions of the two isomeric modifications. The change of α-glucose to β-glucose is reversible and occurs readily, the direction of the change depending on how much of each form is present in the solution. If, on standing, the change α-glucose $\rightarrow$ β-glucose should exceed the transformation β-glucose $\rightarrow$ α-glucose, the optical rotation will diminish. If the reverse should be true, the optical rotation will steadily increase. When equilibrium is reached, i.e., when the specific optical rotation is $[\alpha]_D = +52.5°$, it is found that approximately one-third of the glucose is present as α-glucose and two-thirds as β-glucose.

The α- and β-methyl glucosides are clearly derivatives of the α- and β-forms of glucose, respectively. It is interesting that the average specific optical rotation of the glucosides is +62°, and of the glucoses, +63.5°, a physical relationship that has been ascribed to their structural similarity. When α-methyl glucose is hydrolyzed it yields a sugar having a high initial optical rotation, obviously α-glucose; and when the β-methyl glucoside is hydrolyzed, it yields a sugar of low initial optical rotation, obviously β-glucose.[24]

[24] The molecular rotation of a sugar is the product of its molecular weight and specific rotation (p. 55). Hudson postulated that the molecular rotation depends on two factors: (1) the optical effect of the end asymmetric carbon atom of the sugar, and (2) the optical effect of the remaining asymmetric carbon atoms. If the rotation due to the terminal asymmetric carbon atom of α-*d*-glucose is represented by A and the rotation of the remaining four asymmetric carbon atoms by B, the molecular rotation of the whole molecule is $A + B$. The molecular rotation of the other isomer, β-*d*-glucose, will then be $-A + B$. These facts may be summarized as follows:

Molecular rotation of α-*d*-glucose = $180 \times +110° = 19{,}800 = A + B$

Molecular rotation of β-*d*-glucose = $180 \times +19° = 3{,}420 = -A + B$

Accordingly, the difference in molecular rotation ($2A$) = 16,380 and the sum ($2B$) = 23,220.

Hudson has developed the following generalizations:

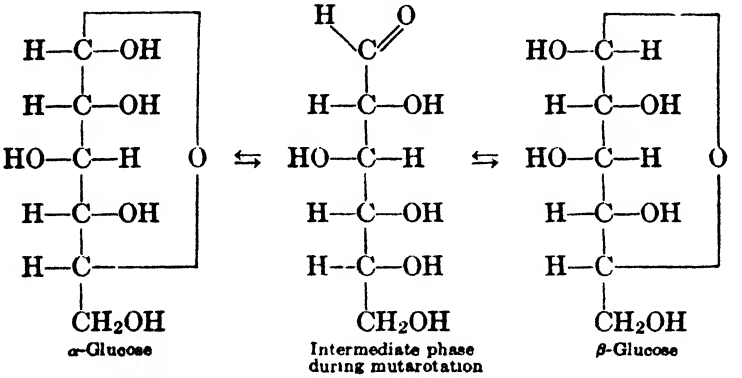
(1) The difference between the molecular rotations of the α- and β-forms of all the aldehyde sugars and all their derivatives in which the added substance is not joined directly to the end asymmetric carbon atom is a nearly constant quantity (about 16,200).

(2) The α- and β-forms of those derivatives (e.g., glucosides, etc.) of any aldose sugar in which only the asymmetric carbon atom is affected have molecular rotations whose sum is equal to the sum for the α- and β-forms of the aldose (approximately 23,000 to 24,000).

(3) The names of the α- and β-forms of the sugars should be so selected that for all sugars which are genetically related to *d*-glucose the subtraction of the rotation of the β-form from that of the α-form gives a positive difference, and for all sugars which are genetically related to *l*-glucose a negative difference.

(4) The names of the α- and β-forms of the derivatives of any sugar should be so

In addition to the cyclic forms of glucose, the existence of a non-cyclic form must be assumed if we are to explain its reactions as an open-chain aldehyde. The opening of the cyclic structure with the formation of the aldehydic form has for many years been associated with the change of one isomeric form of glucose to the other (α-glucose $\rightleftarrows$ β-glucose) which underlies the phenomenon of mutarotation.



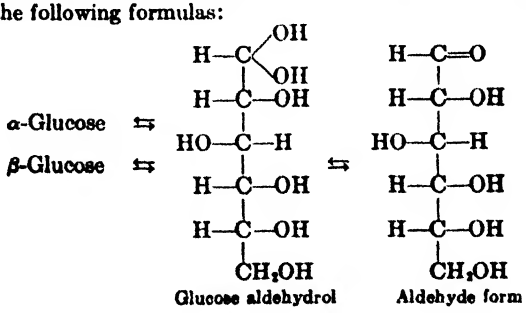
The presence of the transitional aldehyde form is taken as an indication that the sugar is undergoing or has undergone mutarotation. In fact, it has been pointed out (Levene and others) that the sugars or their derivatives which show a higher velocity of mutarotation are also those in which the change to the aldehyde form proceeds with higher velocity.

It was formerly believed by Lowry that the cyclic isomers take up a molecule of water and pass through a transitory aldehydrol stage to the aldehyde form.[25] This view, however, has been challenged by Arm-

selected that the difference of their molecular rotations is equal to and of the same sign as the similar difference for the forms of that glucose (*d*- or *l*-) to which the first sugar is genetically related.

These rules have proved to be very useful in the study of the molecular configuration of the sugars and their derivatives. C. S. Hudson, *J. Am. Chem. Soc.*, **31**, 66 (1909). That the principles involved have certain limitations and may not be applied uniformly to all sugars is suggested by recent work.

[25] If this view were correct, the formation of the aldehyde form of glucose could be represented by the following formulas:



strong [26] on the ground that there is an increase in the conductivity of sugar solutions during mutarotation, a fact which is inconsistent with the formation of an aldehydrol. The chemical mechanism involved in the process of mutarotation has been the subject of intensive study during the last few years, and it seems not unlikely that the phenomenon is purely a tautomeric change and does not depend on the intervention of water. Some of this work has been reviewed by Lowry.[27]

Although it is to be admitted that there is, as yet, no general agreement regarding the nature of the intermediate products which accompany mutarotation, or of the mechanism of their formation, it is probably safe to assume, on the basis of available evidence, that at some stage or another the open-chain aldehyde form of glucose is present. This variety of *d*-glucose is in equilibrium with the α- and β-isomers of the amylene oxide forms and possibly with other cyclic isomeric modifications. When glucose is treated with a reducing or oxidizing agent, with phenylhydrazine, hydrocyanic acid, or any reagent which acts on the free aldehyde group, the equilibrium relations are disturbed by the removal of the aldehyde form from the reacting system. As a result, the α- and β-forms are converted to the aldehyde, and, as the reaction proceeds, more and more of the cyclic forms are changed to the aldehyde form. On the assumption that at any given moment during a reaction only a small amount of the free aldehyde is present, rests the explanation which some have urged for the fact that the sugars are more slowly reactive than are hydroxyaldehydes which do not have a cyclic configuration.

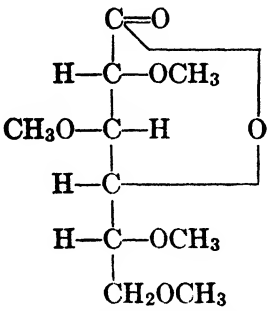
Proof of the Amylene Oxide Formula of Glucose. In describing the cyclic forms of glucose (p. 37) and the corresponding methyl glucosides (p. 34), the δ-, or amylene oxide, structure was assumed. It remains to present some of the evidence upon which this is based.[28]

[26] *J. Chem. Soc.*, **83**, 1305 (1903). See also E. F. Armstrong's "The Carbohydrates and the Glucosides," London, 1924 edition, p. 47.

[27] *Z. physik. Chem.*, **130**, 125 (1927); Lowry and Smith, Dixième conf. union intern. chim., p. 79 (1930).

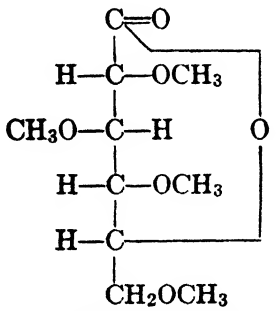
[28] The chemical constitution of the carbohydrates has engaged the attention of numerous investigators for over a generation. Numbered among the pioneer workers in this field were Emil Fischer, J. U. Nef, and E. F. Armstrong, who laid the foundation for the more recent work of J. C. Irvine, C. S. Hudson, P. A. Levene, W. N. Haworth, and others, which has resulted in an almost complete revision of our knowledge of the stereochemistry of the sugars. This subject is a very difficult one, and the methods of investigation are very complex. In gaining an appreciation of the present status of the subject, the student will be aided by the following references: "Progress in the Structural Study of Carbohydrates," J. C. Irvine, *Chem. Rev.*, **4**, 203 (1927); "Active Glucose," P. A. Levene, *ibid.*, **5**, 1 (1928); W. Charlton, W. N. Haworth, and S. Peat, *J. Chem. Soc.*, **129**, 89 (1926); W. N. Haworth and G. C. Westgarth, *ibid.*, p. 880; W. N. Haworth, "The Constitution of Sugars," London, 1929; P. A. Levene and A. L. Raymond, "The Chemistry of the Carbohydrates and Glucosides," *Ann. Rev. Biochem.*, **1**, 213 (1932); **2**, 31 (1933); J. C. Irvine and G. J. Robertson, *ibid.*, **4**, 59 (1935); W. N. Haworth and E. L. Hirst, *ibid.*, **5**, 81 (1936); **6**, 99 (1937).

(1) 2 : 3 : 6 Trimethylglucose gives on oxidation 2 : 3 : 6 trimethylgluconic acid, which readily forms a lactone. This has been identified as a γ-lactone. If this compound is methylated, it yields 2 : 3 : 5 : 6 tetramethylgluconolactone. This is a crystalline solid.



2·3 5 6 Tetramethylgluconolactone

2 : 3 : 6 Trimethylglucose on methylation forms a tetramethylglucose. When this is oxidized it yields a tetramethylgluconolactone, which is a liquid and which in its physical and chemical properties differs markedly from the 2 : 3 : 5 : 6 tetramethylgluconolactone given above. Accordingly, this can only be the 1 : 5 lactone:

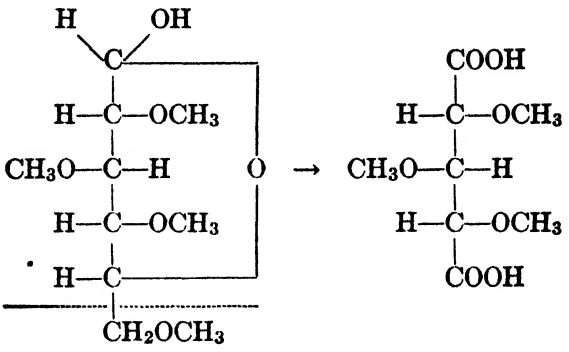


2:3·4:6 Tetramethylgluconolactone

From this it has been concluded that the normal tetramethylglucose (and, hence, glucose itself) is an amylene oxide.[29]

[29] Hudson has expressed doubt of the validity of Haworth's assumption that rings never shift in the methylation of glucosides and glucoside-like derivatives (*J. Am. Chem. Soc.*, **52**, 1680, 1707 [1930]. Hudson's objections have been challenged, however, by Haworth and Hirst (*J. Chem. Soc.*, 2615 [1930], *J. Am. Chem. Soc.*, **52**, 4168 [1930]), and the weight of opinion at present (see Levene and Raymond, *Ann. Rev. Biochem.*, **1**, 213 [1932]) seems to be in favor of the views of the latter school.

(2) Crystalline tetramethylglucose is oxidized by nitric acid to xylotrimethoxyglutaric acid, as follows:



(3) Tetramethylgalactonolactone, derived from tetramethylgalactose, is known to have an amylene oxide configuration. Trimethylarabinose and trimethylxylose are also known to give 1 : 5 lactones. These three lactones have properties which are almost identical with those of the lactone obtained from tetramethylglucose. By analogy, it has therefore been inferred that the tetramethylglucose is also an amylene oxide.

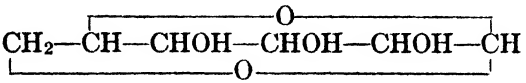
Space Relations of the H and OH Attached to Carbon Atom 1. The positions of the H and OH, attached to carbon atom 1, in the formulas of α- and β-glucose, are not arbitrarily chosen, but are based partly on the following observations:

(1) The conductivity of α-glucose in boric acid solution diminishes during the mutarotation as it is converted to β-glucose, whereas the conductivity of β-glucose is increased under similar conditions as α-glucose is formed. The change in conductivity takes place with the same velocity as the mutarotation, showing that the two phenomena are related. It has been shown that an alcohol increases the conductivity of a boric acid solution if it has two hydroxyl groups attached to two neighboring carbon atoms and situated in the same plane on the same side of the carbon chain. Accordingly, it is concluded that the OH groups attached to carbon atoms 1 and 2 are on the same side in α-glucose and on opposite sides in β-glucose.

(2) On heating α-glucose (150–155° C. under a pressure of 15 mm.), α-glucosan, $CH_2OH—\overset{\displaystyle O}{\overset{\frown}{CH}}—CHOH—CHOH—\underset{\displaystyle O}{\underset{\frown}{CH}}—CH$, is obtained.

This has an ethylene-oxide structure and is formed by the loss of HOH from the two hydroxyl groups attached to carbon atoms 1 and 2. On the assumption that the hydroxyls are able to react with one another only when they are on the same side of the carbon chain, it is inferred that the

OH group attached to carbon atom 1, in α-glucose, occupies the position as represented by the formula on p. 37. β-glucose yields β-glucosan,[30]



Cyclic Forms of Glucose, Other Than the Amylene Oxide. "Active Glucose." The stable form of glucose, as well as of mannose, galactose, fructose, xylose, arabinose, and probably other sugars, in aqueous solution, is the amylene oxide form, but it is unlikely that this is the only cyclic structure which glucose (and the other sugars) may possess. Indeed, a considerable amount of evidence points to the existence of a so-called "active" (or reactive) glucose which is not an amylene oxide compound.

As to the nature of the "active" glucose, there is some basis for the view that it is the γ-, or butylene oxide, form. Levene [31] is of the opinion, however, that in aqueous sugar solutions, all theoretically possible cyclic structures may co-exist in equilibrium with one another (i.e., the α- and β-modifications of the ethylene oxide, the α- and β-modifications of the propylene oxide, as well as the two isomers of the butylene oxide and amylene oxide forms of glucose). The amylene oxide, being the most stable, predominates. The other forms may be present, usually in insignificant amounts, but are brought into existence individually by agents which act specifically or preferentially on one lactal form and none other.

In considering the nature of "active" glucose the remarkable instability exhibited by sugars in alkaline solution cannot be neglected. Sugars are weak acids, and as such form ionizable salts with base. It is to this property that Shaffer and Friedemann [32] have attributed the instability and reactivity of the sugars. That the "active" forms of the sugars may be *sugar ions* is a significant concept. The action of alkali on glucose and other sugars will be dealt with shortly.

The main conclusion to be derived from the discussion in the preceding paragraphs is that glucose in solution does not behave as though it were a compound having a fixed molecular structure. On the contrary, it is to be regarded as existing in at least five and possibly in nine different forms, one of which is represented as the open-chain aldehyde, and the other eight possessing cyclic configurations (ethylene oxide,

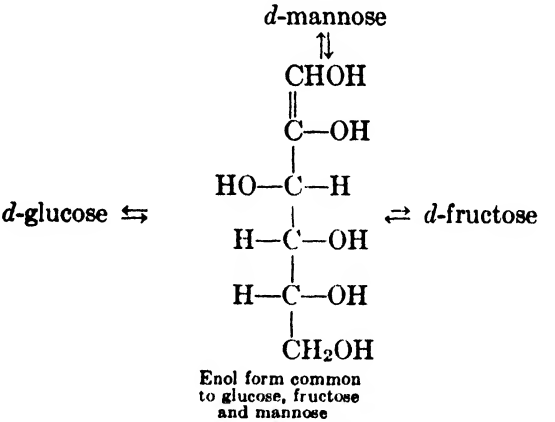
[30] For a more complete and more critical discussion of this question, see Armstrong's "The Carbohydrates and Glucosides," p. 44. See, also, P. A. Levene and H. Sobotka, *J. Biol. Chem.*, **67**, 759 (1926). Reference may also be made to the important papers of J. Böeseken and associates, *Ber.*, **46**, 2612 (1913); *Proc. Roy. Acad. Amsterdam*, **18**, 1654 (1916); *Rec. trav. chim.*, **40**, 354 (1921); and to the paper of M. Levy and E. A. Doisy, *J. Biol. Chem.*, **84**, 749 (1929).

[31] *Chem. Rev.*, **5**, 1 (1928).

[32] *J. Biol. Chem.*, **86**, 345 (1930).

propylene oxide, butylene oxide, and amylene oxide).[33] Of these the amylene oxide form predominates, the others usually being present in very small amounts. The various forms are in equilibrium with one another, and under appropriate conditions are convertible one into another. Nor does this concept apply only to glucose. It is very likely that what has been said with regard to this sugar applies with equal force to the other simple carbohydrates.

The Action of Alkali on Glucose and Other Monosaccharides. If *d*-glucose is treated with a dilute basic solution, such as 0.05 *N* $Ca(OH)_2$, it changes its optical rotation, which ultimately reaches an equilibrium. The equilibrium mixture contains *d*-glucose, *d*-fructose, and *d*-mannose, as well as small amounts of *d*-glutose, and *d*-pseudofructose.[34] This conversion, first studied by Lobry de Bruyn,[35] also occurs if *d*-fructose or *d*-mannose is treated with alkali, the final products being the same as with glucose. It seems that, in these interconversions, the α- and β-carbon atoms are involved. It is believed that glucose, fructose, and mannose are capable of forming an enol which is common to all, and that from this enol the three sugars are regenerated. The relationship may be represented as follows:



In more concentrated alkaline solutions, the sugars are oxidized spontaneously in the presence of air, with the formation of a large number of simpler compounds. Nef [36] was of the opinion that from glucose at least 93 substances are formed in this way, and possibly as many

[33] If the α- and β-isomers of the hexylene oxide, ϵ or $< 1 : 6 >$ form, the existence of which is questioned, were included, the total number of glucose isomerides would be eleven.

[34] Glutose, when represented in the non-cyclic form, is $CH_2OH—CHOH—CO—CHOH—CHOH—CH_2OH$. Pseudofructose differs from fructose (p. 48) in the β-carbon atom configuration.

[35] *Rec. trav. chim.*, **14**, 156, 203 (1895).

[36] *Ann. Chem.*, **357**, 214 (1907); **403**, 204 (1913).

as 116 if those compounds are included which result from the resynthesis of the initial fragments of glucose disintegration.

The acidic property of sugars has been a subject of investigation in Shaffer's laboratory.[37] For glucose, fructose, and sucrose, Urban and Shaffer have recently obtained the following values for the first two dissociation constants, at 25° C.:

	pK_1	pK_2
Glucose	12.09	13 85
Fructose	11 68	13 24
Sucrose	12.60	13 52

The existence of a third acidic group has been considered, but Urban and Williams were unsuccessful in obtaining evidence of a third dissociation constant below *p*H 13.6, for any of the sugars studied, with the possible exception of lactose.

It is thus seen that a moderate alkalinity is required to bring out the first acidic group and a high alkalinity the second and third, assuming the last to exist. And, as has been stated previously, it is in the presence of sufficient alkali to form salts that the sugars acquire their greatest instability and reactivity. This has been clearly brought out in a series of remarkable experiments by Shaffer and Friedemann.[32] These investigators determined the relation of the degree of alkalinity to the non-oxidative transformation of various sugars into the so-called saccharinic acids (C_3, C_4, etc., acids) and lactic acid. They found that a high alkalinity favored the yield of lactic acid, while low alkalinity or a high sugar concentration and a high temperature lowered the yield.

The Structural Relationship of the Monosaccharides. Two methods have been especially valuable in studying the structural relationship of the simple sugars. The first method is the so-called cyanhydrin synthesis of Kiliani, which is based on the reaction of aldehydes and ketones with HCN. If a sugar, such as *d*-glucose, is treated with HCN, the corresponding nitrile is formed, which on hydrolysis yields an acid containing one carbon atom more than the original sugar—in this case a seven-carbon sugar acid (glucoheptonic acid). On reduction, the acid is converted to the aldehyde, glucoheptose.[38] By this method it is pos-

[37] *J. Biol. Chem.*, **94**, 697 (1932); **100**, 237 (1933).

[38] $\underset{\text{Glucose}}{C_6H_{12}O_6} + HCN = \underset{\substack{\text{Nitrile of}\\ \text{glucoheptonic acid}}}{C_6H_{13}O_6\cdot CN};$

$$C_6H_{13}O_6\cdot CN + 2H_2O = \underset{\text{Glucoheptonic acid}}{C_6H_{13}O_6\cdot COOH} + NH_3;$$

$$C_6H_{13}O_6\cdot COOH \xrightarrow{-O} \underset{\text{Glucoheptose}}{C_7H_{14}O_7}.$$

sible to produce from *d*-glycerose the two four-carbon monosaccharides, *d*-erythrose and *d*-threose. From these the four *d*-pentoses may be synthesized and, in turn, the eight *d*-hexoses. Continuing the cyanhydrin synthesis, it is possible to prepare heptoses, octoses, and even higher monosaccharides.

The second method is that of Wohl, which depends on the reaction of aldehydes and ketones with hydroxylamine with the formation of oximes. From glucose, glucose oxime is formed. When this is treated with concentrated NaOH, it yields the nitrile of gluconic acid, which, on heating, decomposes to HCN and arabinose.[39] This method of degradation of sugars, together with Kiliani's method of synthesis, has been especially useful in determining the stereoisomeric relationship of the sugars.

It may be pointed out again that the *d* and *l* nomenclature of the sugars is not based on the direction of their optical rotation, but on their relationship to *d*- and *l*-glycerose, or to *d*- and *l*-glucose. On p. 47, this relationship is shown by means of formulas, the arrangement being based on Emil Fischer's classification. The members of the *l*-series, derived from *l*-glycerose, may be represented in a similar way.

Hexose Sugars. Of the hexoses, those that are found free in nature are *d*-glucose and *d*-fructose. The former is widely distributed in fruit and plant juices (grape, sweet corn, onions, unripe potatoes). It is also referred to as grape sugar, or dextrose. Glucose is a normal constituent of the blood and is utilized by the tissues in the production of energy. It may be obtained readily by enzymic or acid hydrolysis of maltose, lactose, sucrose, dextrin, starch, glycogen, and cellulose. The products of sucrose hydrolysis are fructose and glucose. Sucrose is dextrorotatory $[\alpha]_D = +\ 66.5°$. As fructose is more strongly levorotatory, the mixture obtained after sucrose is hydrolyzed is levorotatory $[\alpha]_D = -\ 19.84°$. The term "invert sugar" has therefore come to be used in describing the mixture of glucose and fructose resulting from the hydrolysis, or "inversion," of sucrose.

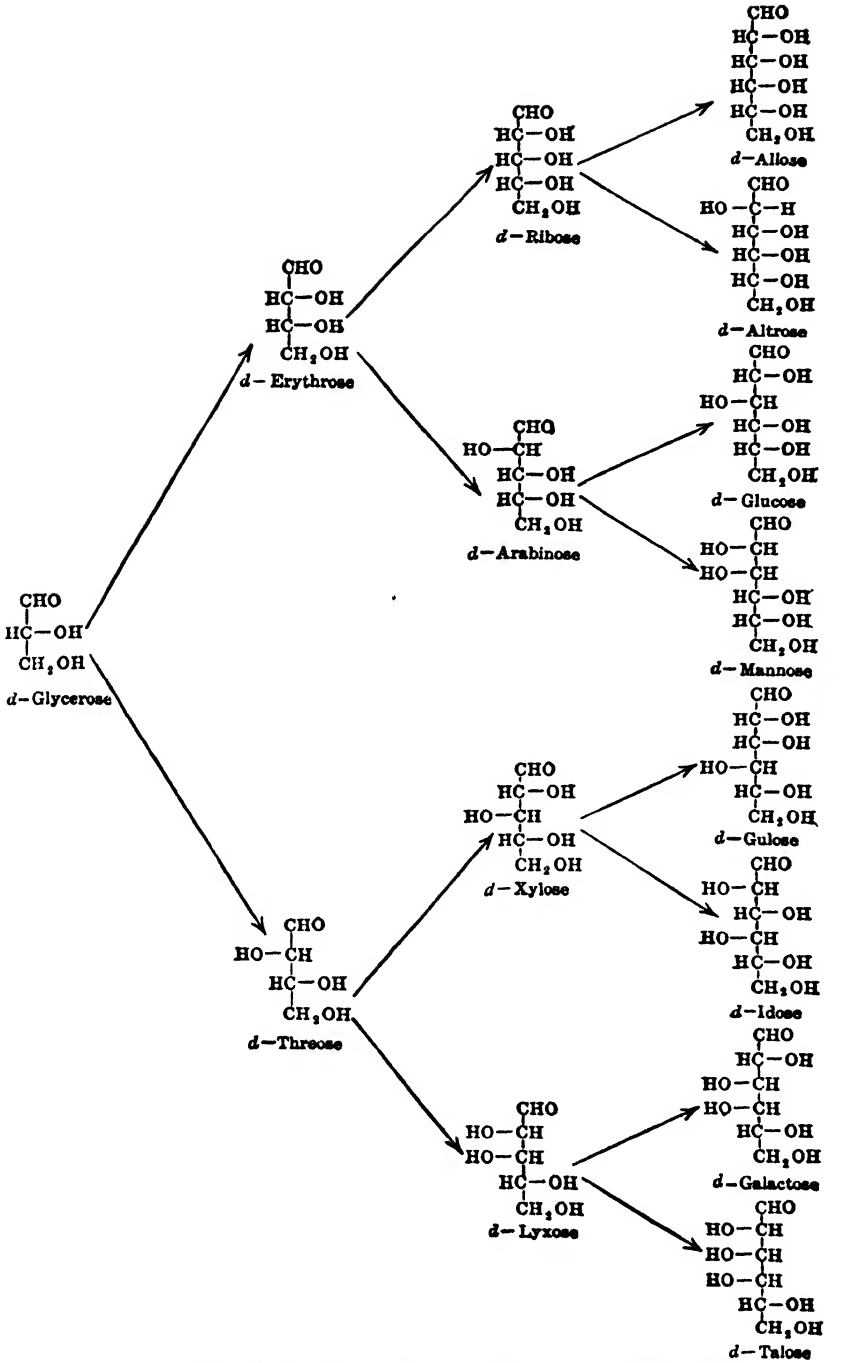
Fructose may be prepared readily by acid hydrolysis of inulin. Together with glucose it occurs in fruit juices and in honey. From "invert sugar," the ordinary crystalline glucose and fructose are isolated. The latter is believed to be an amylene oxide compound (Haworth and Hirst).[40] It seems, however, that, as it exists in the sucrose molecule, the fructose is in the labile or γ-form. The γ-fructose itself has not been isolated, but when heptamethyl sucrose is hydrolyzed, the fructose component is obtained in the form of tetramethyl-γ-fructose. Accord-

[39] $\underset{\text{Glucose}}{C_5H_{11}O_5\cdot CHO} + \underset{\text{Hydroxylamine}}{NH_2OH} = \underset{\text{Glucose oxime}}{C_5H_{11}O_5\cdot CH:NOH} + H_2O;$

$C_5H_{11}O_5\cdot CH:NOH + NaOH = \underset{\text{Nitrile of gluconic acid}}{C_5H_{11}O_5\cdot CN} + NaOH + H_2O;$

$C_5H_{11}O_5\cdot CN \xrightarrow{\text{heat}} \underset{\text{Arabinose}}{C_5H_{10}O_5} + HCN.$

[40] *J. Chem. Soc.*, **129**, 1858 (1926).

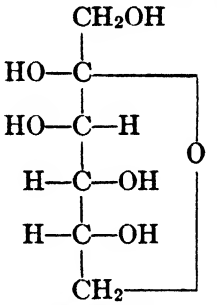


Tetrose, Pentose, and Hexose Sugars Derived from *d*-Glycerose

ingly, it has been assumed that, immediately on its liberation from its union with glucose, the labile variety passes into the more stable δ-variety of fructose. This may be represented as follows:

Sucrose → glucose (normal) + fructose (labile or γ)
↓
crystalline fructose (normal)
on isolation

The normal variety of fructose, of which there is, of course, both an α- and a β-form, is represented as having an amylene oxide configuration:



Fructose (β-Form)

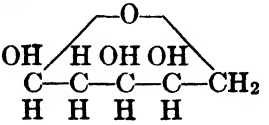
d-Galactose is the constituent sugar of the polysaccharides which are classified as galactans. These are widely distributed in plants, being especially abundant in algae and lichens, including agar-agar and Irish moss. By hydrolytic methods, galactose may be conveniently prepared from these substances, as well as from the wood of the western larch. Galactose also occurs in saponins. It is a constituent sugar of the disaccharide lactose (milk sugar) and is likewise a component of certain fatlike substances, present in brain tissue, known as cerebrosides (p. 77). Snails and especially the eggs of snails are said to contain a galactogen, an analogue of glycogen.

Mannose is the constituent sugar of the mannans, a group of polysaccharides widely distributed in plants, but especially abundant in the seed of the tagua palm, better known as vegetable ivory, from which buttons are made. Levene and Mori [41] have reported mannose to be a constituent of ovomucoid, a conjugated protein (p. 83) which occurs in egg white.

Pentose Sugars. The pentoses have been detected, in small amounts, in the free form, in certain plants, but for the most part they occur in polysaccharide combination as pentosans. Cherry gum yields *l*-arabinose. Hydrolysis of straw, hay, oat hulls, corncobs, and most

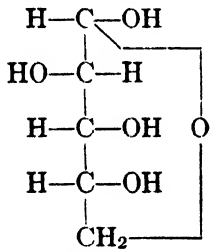
[41] *J. Biol. Chem.*, **84**, 49 (1929). The occurrence of mannose in association with proteins has also been reported by Fränkel and Jellinek (*Biochem. Z.*, **185**, 392 [1927]) and by Rimington (*Biochem. J.*, **23**, 430 [1929]); see also M. Sørensen and G. Haugaard, *Compt. Rend. trav. Lab. Carlsberg*, **19**, No. 12 (1933).

woods yields *d*-xylose. *d*-Ribose is a normal constituent of the mononucleotides, guanylic and inosinic acids, as well as of plant nucleic acid. The sugar in animal nucleic acid (thymonucleic acid) is a desoxyaldopentose, namely, *d*-2-ribodesose.

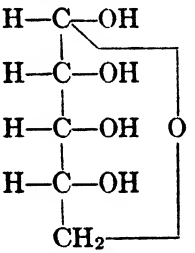


A pentose is present in the urine in the relatively rare condition known as pentosuria. Various workers have been unable to agree regarding the kind of pentose, some considering it to be arabinose, others ribose, still others xylose, etc. Greenwald [42] identified *l*-xyloketose in four cases and Enklewitz and Lasker [43] in thirty-eight cases of pentosuria, and it is therefore not improbable that this is the only pentose found in the urine in this condition. A possible origin of *l*-xyloketose will be indicated elsewhere (p. 339).

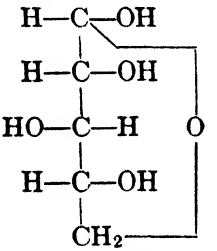
Structurally, the aldopentoses, in their stable form, are believed to have the amylene oxide configuration.



d-Arabinose



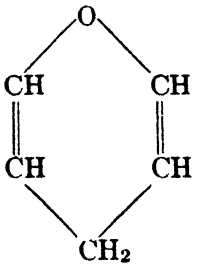
d-Ribose



d-Xylose

(α-Forms)

Relation of the Sugars to Pyran, the Pyranose Series. As a further development in our conception of the molecular configuration of the normal sugars, Haworth [44] introduced the generalization that they are structurally related to pyran:

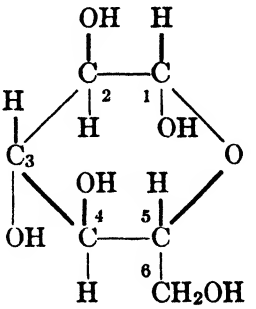


[42] *J. Biol. Chem.*, **89**, 501 (1930).

[43] *Ibid.*, **110**, 443 (1935).

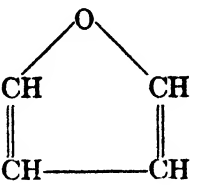
[44] W. N. Haworth, "The Constitution of Sugars," Edward Arnold Co., London, 1929.

On examining the formulas representing the stable forms of glucose (p. 37), fructose (p. 48), and the pentoses (p. 49), it will be observed that these contain a six-atom ring, five of the atoms being C, the sixth O. The six-atom ring is appropriately represented as a hexagon, and, in the formulation of the normal sugars, the addenda (hydrogen atoms, hydroxyl and primary alcohol groups) may be arranged in accordance with the available knowledge of the constitution of the respective sugar. Accordingly, *d*-glucose, (β-form) may be represented as follows:

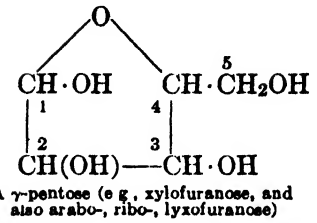


Indeed, Haworth has recommended the revision of the present nomenclature of the sugars and the adoption of a terminology which would distinguish the normal, or δ sugars as *pyranoses* because of their relation to pyran. Accordingly, arabinose, xylose, and ribose may be designated as *arabopyranose*, *xylopyranose*, and *ribopyranose*, respectively. Similarly, glucose, galactose, mannose, and fructose may be described as *glucopyranose*, *galactopyranose*, *mannopyranose*, and *fructopyranose*.

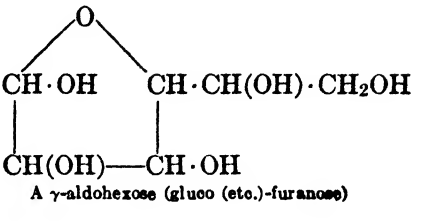
Relation of the Labile or γ-Sugars to Furan, the Furanose Series. The γ-oxide forms of glucose, fructose, and other sugars have not been isolated, but certain of their derivatives have been prepared and studied. Sufficient evidence has accumulated to show that these have a five-atom ring structure. They, and presumably, therefore, the sugars from which they are derived, may be considered as being related to furan:



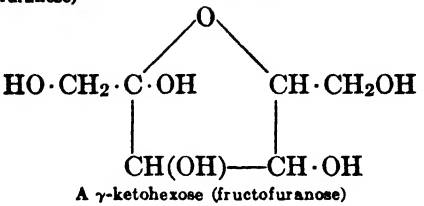
Analogous to the description of the normal or amylene oxide forms of the sugars as pyranoses, the γ-oxide forms may be designated as *furanoses*.



A γ-pentose (e g , xylofuranose, and also arabo-, ribo-, lyxofuranose)



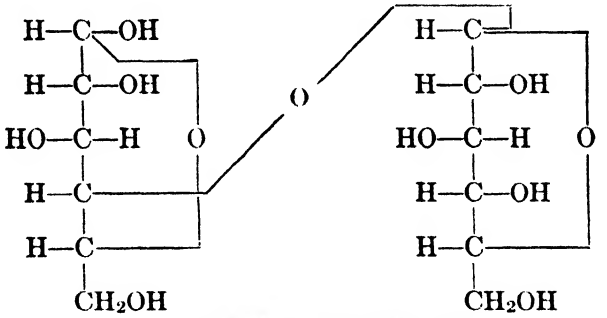
A γ-aldohexose (gluco (etc.)-furanose)



A γ-ketohexose (fructofuranose)

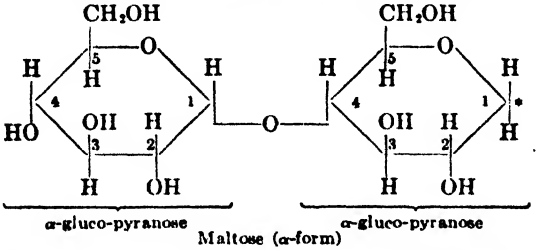
Occurrence and Constitution of the Disaccharides. The physiologically important dissacharides are maltose, lactose, and sucrose.

Maltose (malt sugar) may be formed by incomplete hydrolysis of starch. It is a constituent of germinating cereals and malt. The sugar crystallizes in small needles, has reducing properties, is fermented by yeasts, forms an osazone with phenylhydrazine, and exhibits mutarotation. Maltose is α- or β-glucose-α-glucoside, and, on hydrolysis by acid or the enzyme maltase, yields glucose. It is represented stereochemically by the following formula: [45]



α-Maltose (α-glucose-α-glucoside)

[45] The molecular configuration of maltose may also be represented by the formula:

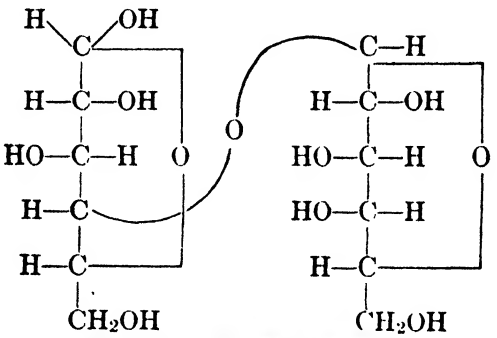


Maltose (α-form)

The free reducing group of the disaccharide is indicated by the asterisk.

Two isomeric modifications, α-maltose (α-glucose-α-glucoside) and β-maltose (β-glucose-α-glucoside), are possible, depending on the arrangement of the H and OH attached to carbon atom 1 of the glucose portion of the molecule.

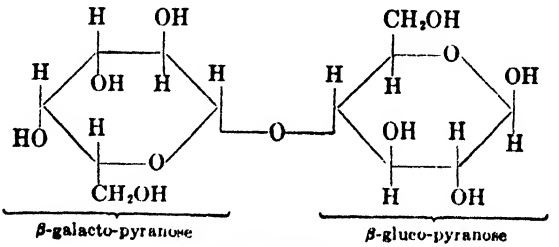
Lactose (milk sugar) occurs in milk. It is hydrolyzed to glucose and galactose either by acid or by the enzyme lactase. Lactose is a reducing sugar, forms an osazone when treated with phenylhydrazine, and exhibits mutarotation. Therefore, it must have a free or a potentially free aldehyde group and should be present in at least two isomeric forms. Lactose is believed to have the following molecular configuration: [46]



Lactose (α- or β-glucose-β-galactoside)

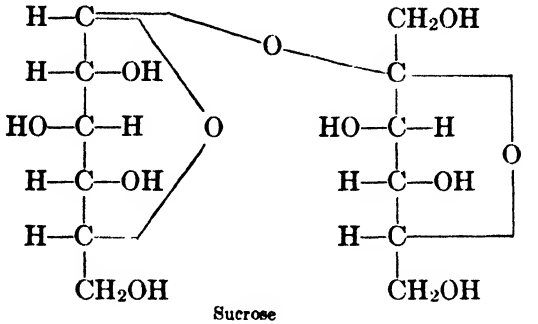
Sucrose (saccharose, cane sugar) occurs in the sugar beet, sorghum cane, sugar maple, pineapple, in the roots of carrots, and in many other plants. It is fermented by yeast, the first step being the inversion of the sucrose by the enzyme invertase which is present in the yeast. Sucrose has no reactive aldehyde group, is therefore non-reducing, does not form an osazone, and does not exhibit mutarotation. On hydrolysis it yields fructose and glucose. As has been pointed out previously (p. 46), the fructose in the sucrose molecule is believed to have the γ-oxide ring

[46] Lactose may also be represented by the following formula:



Lactose (β-form)

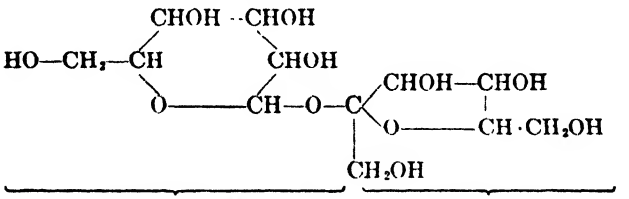
(fructofuranose). Sucrose may be represented by the following structural formula:[47]



Sucrose

A number of disaccharides have been obtained from naturally occurring trisaccharides. From *gentianose*, a trisaccharide (glucose-glucose-fructose) present in the roots of the gentian, *gentiobiose* (glucose-β-glucoside) has been obtained. It is stated that, in the acid hydrolysis of starch, 5.7 per cent of it is converted into gentiobiose. This disaccharide is also formed when *d*-glucose is acted on by strong hydrochloric acid. It is apparently identical with "isomaltose," which Emil Fischer obtained by the condensation of glucose. *Cellobiose*, or cellose (glucose-β-glucoside) is obtained by the partial hydrolysis of either cotton or wood cellulose.[48] *Melibiose* (glucose-β-galactoside) is formed from

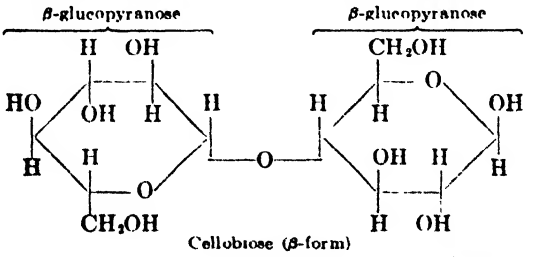
[47] Sucrose may also be represented by the formula:



Glucopyranose Fructofuranose

Sucrose

[48] Both gentiobiose and cellobiose are glucose-β-glucose. The difference in the chemical constitution of the two sugars is indicated by the following formulas:



Cellobiose (β-form)

(*Footnote continued on page 54*)

raffinose (fructose-glucose-galactose) by incomplete hydrolysis. Raffinose occurs in the sugar beet. *Turanose* (fructose + glucose) is obtained by hydrolyzing the trisaccharide *melezitose* or *melicitose* (glucose-glucose-fructose). Melezitose is a constituent of the sap of certain trees, such as the larch, scrub pine, and Douglas fir.

The Specific Optical Rotation of Various Sugars. Owing to the presence of asymmetric carbon atoms, the sugars have the property of rotating the plane of polarized light. The specific rotatory power, or specific rotation, determined at 20° C. in sodium light (D line) may be computed from the formula:

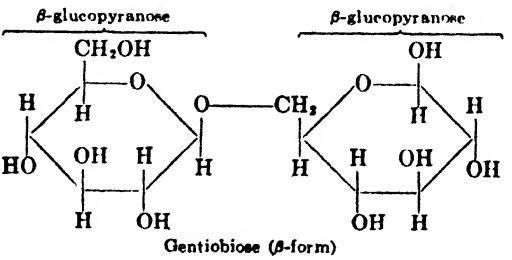
$$(\alpha)_{D}^{20^\circ} = \frac{a \times 100}{lc}$$

in which a = observed rotation, l = length of the polariscope tube in decimeters, and c = number of grams of optically active substance per 100 cc. The specific rotation (a) of a given compound is defined as the angle of rotation through which a plane of polarized light (the source of illumination is sodium light) is turned in passing through a tube 1 decimeter in length, filled with a solution containing 1 gram of the substance to 1 cc. Both the temperature, 20° C., and the source of illumination, sodium light (D line), are stated in the formula.

Determinations have been made of the specific rotation of a large variety of substances. For sucrose, the value is +66.5°. Special types of the polariscope, called saccharimeters, have been designed for use in sugar analyses. When the specific rotation of a given compound is known, its concentration in a solution of unknown strength may be determined from the observed angle of rotation which is produced by the solution.

In Table VIII are given the specific optical rotations of a number of mutarotating sugars. It will be recalled that the shift in optical rotation which is exhibited by a sugar such as glucose, galactose, maltose, etc., in solution, is due to the conversion of one isomeric modification to another.

Sweetness of Sugars. Fructose is the sweetest of the common sugars. Sucrose is sweeter than glucose; lactose has approximately 16 per cent the sweetening power of sucrose. Values for the relative



Gentiobiose (β-form)

TABLE VIII

SPECIFIC ROTATION IN WATER OF MUTAROTATING SUGARS

(After Abderhalden)*

Sugar	α-Form	Equilibrium	β-Form
d-Glucose	+113.4°	+52.2°	+19°
d-Galactose	+144	+80.5	+52
d-Mannose	+34	+14.6	−17
d-Fructose	−21	−92	−133.5
d-Xylose	+92	+19	−20
d-Arabinose	−54	−105	−175
Lactose	+90	+55 3	+35
Maltose	+168	+136	+118

* Taken from data in "Biochemisches Handlexikon," Vol. 10, p. 366.

sweetness of pure sugars as estimated by Biester, Wood, and Wahlin,[49] are given in Table IX.

TABLE IX

RELATIVE SWEETNESS OF VARIOUS SUGARS

(Sucrose = 100)

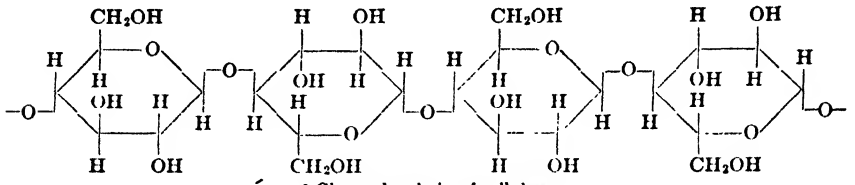
Fructose	173 3	Maltose	32 5
Sucrose	100 0	Rhamnose	32 5
Invert sugar	130 0	Galactose	32 1
Glucose	74 3	Raffinose	22 6
Xylose	40 0	Lactose	16 0

THE POLYSACCHARIDES

Cellulose. Cellulose is the term applied to a class of compounds which in common with starch and glycogen may be designated by the empirical formula $(C_6H_{10}O_5)_x$ and classified as hexosans or glucosans. On complete hydrolysis, cellulose, from whatever source, yields glucose. In controlled hydrolyses, the disaccharide cellobiose may be obtained. Cellulose occurs in the fibrous or supporting tissues of plants and in the walls of plant cells. It occurs in relatively pure form in cotton. Cellulose is the principal constituent of wood, where it occurs in combination with a substance called lignin. Lignin is partly composed of pentosans. The cementing material which holds plant cells together is a cellulose-pectin complex (pectocellulose, or protopectin). With the exception of the covering of tunicates, cellulose is found exclusively in plants.

[49] *Am. J. Physiol.*, **73**, 387 (1925); J. J. Willaman, C. S. Wahlin, and A. Biester, *ibid.*, p. 397. For comparison with other data, consult International Critical Tables, Vol. I, p. 357.

On the basis of the X-ray studies of Sponsler and Dore [50] and other data, the cellulose molecule is believed to be composed of a bundle of long chains of chemically united β-glucose (β-pyranose) residues. The individual glucose units are probably joined to each other as in cellobiose, that is, between position 4 of one glucose residue and the reducing group of the adjacent unit. This is indicated by the following formula representing a portion of the cellulose molecule.



β-Glucosidic chain of cellulose

The molecular weight has not been established with certainty, but, according to the measurements of one group of investigators (Stamm,[51] Kraemer and Lansing [52]), the cellulose molecule is composed of nearly 2000 glucose units, from which the molecular weight is estimated to be about 300,000.

Cellulose is relatively resistant to the action of mild chemical reagents. Weak acids and alkalies produce very little effect. In higher concentrations, acids hydrolyze cellulose. Alkali gelatinizes and renders it translucent. Many valuable commercial products are derived from cellulose: paper, mercerized cotton, artificial silk, celluloid, collodion, cellophane, pyroxylin lacquers, varnishes, leather substitutes, motion-picture films, guncotton, etc.

Herbivorous animals, particularly the ruminants, utilize a considerable proportion of the cellulose ingested. In man it is of no importance as food, although it is stated that young and tender cellulose, present, for example, in cabbage and lettuce, is utilized to some degree. The chief value of cellulose in the human dietary is ascribed to the fact that it provides "bulk" to the intestinal contents, thereby facilitating peristalsis and the elimination of food residues.

Starch. This is the principal form in which carbohydrate is stored in the plant and is especially abundant in cereals, seeds, bulbs, and tubers. In some plants (apple, banana), during the ripening process, starch is converted into glucose; in others (corn, peas, etc.), the reverse occurs, namely, conversion of sugar into starch, a process which seems to require the presence of potassium. In the plant, the starch occurs in

[50] "Colloid Symposium Monograph," Vol. 4, 174 (1926), Chemical Catalog Co., New York. Consult also review by Sponsler and Dore, "X-Ray Studies on the Structure of Compounds of Biochemical Interest," *Ann. Rev. Biochem.*, **5**, 63 (1936).

[51] *J. Am. Chem. Soc.*, **52**, 3047, 3062 (1930).

[52] *Nature*, **133**, 870 (1934).

granules which have concentric stratifications. These are characteristic for any given species of plant.[53]

In the raw state, starch is not soluble in cold water, nor is it readily digested by starch-splitting, or amylolytic, enzymes. This is due to the resistance of the outer layer of the starch granule. Starch is a hydrophilic colloid and may take up a considerable amount of water. When it is heated in water, the granules swell but do not necessarily rupture. Prolonged heating or fine grinding may cause the disintegration of starch granules. After raw starch has been subjected to grinding in a ball mill a portion will go into solution in cold water.

When treated with iodine, most starches give an indigo-blue color. Among the first products which starch yields on hydrolysis are the dextrins. The more complex dextrins, when treated with iodine, give colors varying from purple in the case of amylodextrin to a reddish brown color given by erythrodextrin. The simpler dextrins (achroödextrin) yield no color when treated with iodine. The final product of the enzymic hydrolysis of starch by amylase is maltose. It requires the action of the enzyme maltase to complete the hydrolysis to the glucose stage. The conversion of the starch to glucose is not quantitative, however. An unfermentable residue of gentiobiose remains. Starch is one of the most important constituents of the human diet, constituting 50 to 70 per cent of the solid matter of most cereal grains and about 80 per cent of the solids of the potato. It is of interest that a small amount of phosphorus exists in ester combination in starch; the content in potato starch has been found to be about 0.06 per cent.

Starch is believed to consist of at least two substances, *amylose* and *amylopectin*. A third constituent associated with many starches is *hemicellulose*. There is much confusion about the chemical and physical properties and also the terminology of amylose and amylopectin. It is possible that the method of preparing starch may affect the properties of the two fractions, but it has been stated that the only difference between amylose and amylopectin is that the latter is the phosphoric acid ester of the former. When potato starch is treated with acid-ethyl alcohol it loses its amylopectin properties and is apparently converted into amylose. The change occurs without degradation of the starch molecule and without loss of any of the phosphoric acid originally present in the starch.

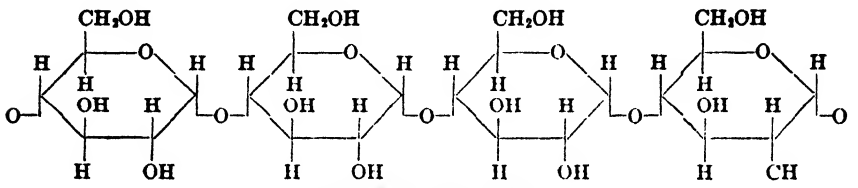
The size of the starch molecule is said to be essentially of the same order of magnitude, irrespective of the origin of the starch. The more recent determinations of Haworth and collaborators [54] indicate that the

[53] The microscopy of starches is comprehensively described in E. T. Reichert's "The Differentiation and Specificity of Starches in Relation to Genera, Species, etc. Stereochemistry Applied to Protoplasmic Processes and Products, and as a Strictly Scientific Basis for the Classification of Plants and Animals," Parts I and II, Carnegie Institution of Washington, Pub. 173, Washington, D. C., 1913.

[54] References to the recent literature will be found in the reviews by W. N. Haworth and E. L. Hirst, *Ann. Rev. Biochem.*, **6**, 99 (1937), and by E. L. Hirst and S. Peat, *Ann. Repts. Chem. Soc., London*, **32**, 285 (1935).

starch molecule is composed of 24 to 26 α-glucose (α-glucopyranose) units. The molecular weight of starch is therefore approximately 4000. It is remarkable that the separate fractions, amylose and amylopectin, are similarly constituted. α-Amylodextrin, a product of starch hydrolysis, consists of a chain of about 17 glucose residues, while, among the simpler dextrins that have been obtained on heating starch with glycerol, one was found to consist of only 7 glucose units.

The glucose units in the starch molecule are joined together, as in maltose, between position 4 of one α-glucose residue and the reducing group of the next unit. This is indicated by the following formula representing a portion of the starch molecule.



Glycogen, the reserve carbohydrate of the animal body, is stored principally in the liver and muscles. It is widely distributed in the animal kingdom, being especially abundant in molluscs, echinoderms, and other invertebrates. Certain fungi and yeasts likewise contain glycogen. In these plants, chlorophyll is lacking, a fact which may point to a mechanism of carbohydrate synthesis different from that in most plants. When purified, glycogen is a white amorphous powder, odorless and tasteless, having many properties in common with starch. Dissolved in water, it gives an opalescent colloidal solution. Iodine colors glycogen red-brown or deep red. Glycogen is apparently acted upon by starch-splitting enzymes.

Glycogenesis, or the synthesis of glycogen, takes place very rapidly in the animal body. The reverse process, glycogenolysis, is likewise a rapid one, especially when the tissues require carbohydrate for combustion. These transformations will be considered in greater detail elsewhere. Acid or enzyme hydrolysis of glycogen yields glucose as the final product, the intermediate products formed resembling the dextrins obtained from starch.

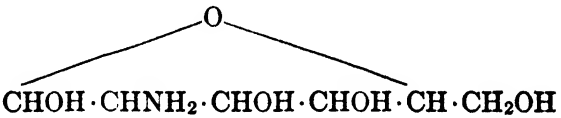
The glycogen molecule is believed to consist of a chain of 12 or 18 α-glucose units.

Immunologically Specific Polysaccharides. Soluble polysaccharides have been isolated from certain bacterial cultures which react with the antisera of the respective bacteria to form precipitates. For example, the polysaccharides obtained from fluid cultures of the various types of pneumococci, in dilutions as high as 1 : 6,000,000, have been found to be specific precipitants for the antisera of the correspond-

ing organisms. Heidelberger and Goebel [55] hydrolyzed the polysaccharide from type III pneumococcus and obtained aldobionic acid, $C_{11}H_{19}O_{10}\cdot COOH$, which on further hydrolysis yielded glucose and glycuronic acid. The molecular weight of the polysaccharide has been estimated to be about 18,000 (Babers and Goebel [56]). A polysaccharide, to which the empirical formula $(C_{30}H_{44}O_{26})_x$ has been assigned by Goebel, is said to occur in type A Friedlander's bacillus. On hydrolysis, this polysaccharide yields aldobionic acid, glucose, and a disaccharide of unknown constitution.

The tubercle bacillus has also been found to contain a specific polysaccharide. On hydrolysis it yields *d*-mannose, *d*-arabinose, and possibly galactose and other substances. Specific polysaccharides are evidently produced also by *Streptococcus viridans*, gonococcus, meningococcus, dysentery bacillus (Shiga), and other microörganisms.

Hexosamines. Chitin, the outer covering of insects and Crustacea yields on hydrolysis an amino hexose (hexosamine), called chitosamine. It has been identified as 2-aminoglucose.



Glucosamine is also a constituent of certain proteins. Egg protein contains a trisaccharide composed of 2 molecules of mannose and 1 molecule of glucosamine.[57] A glucosamine dimannoside has also been isolated from horse serum protein (Rimington [58]).

Mucoproteins (mucins, mucoids, glycoproteins) are classified as conjugated proteins (p. 83); they are characterized by having a prosthetic group, mucoitinsulfuric acid, the components of which are: sulfuric acid, acetic acid, hexosamine and hexuronic (glycuronic) acid. Closely related to mucoitinsulfuric acid is chondroitinsulfuric acid. This occurs in chondroproteins (present in cartilage), as well as in mucoproteins. On hydrolysis, chondroitinsulfuric acid yields sulfuric acid, acetic acid, hexuronic acid, and a hexosamine which is apparently galactosamine (also called chondrosamine).[59]

[55] M. Heidelberger, *Chem. Rev.*, **3**, 403 (1927); Heidelberger and W. F. Goebel, *J. Biol. Chem.*, **70**, 613 (1926); *ibid.*, **74**, 613 (1927). Goebel, *J. Biol. Chem.*, **74**, 619 (1927); **89**, 395 (1930); Heidelberger, F. E. Kendall, and H. W. Scherf, *J. Expl. Med.*, **64**, 557 (1936).

[56] *J. Biol. Chem.*, **89**, 387 (1930).

[57] S. Frankel and C. Jellinek, *Biochem. Z.*, **185**, 392 (1927); P. A. Levene and T. Mori, *J. Biol. Chem.*, **84**, 49 (1929); P. A. Levene and A. Rothen, *ibid.*, **85**, 63 (1929).

[58] *Biochem. J.*, **23**, 430 (1929).

[59] For further details concerning this interesting group of compounds the student is referred to the monograph by P. A. Levene, "Hexosamines and Mucoproteins," Longmans, Green & Co., New York, 1925.

Recent studies by Karl Meyer [60] and others have contributed much to our rather meager knowledge of the occurrence and chemistry of these compounds. From pig gastric mucin, Meyer and co-workers isolated two different polysaccharides, one acid, the other neutral. The acid compound contained one molecule of glycuronic acid, one of acetylglucosamine and one of sulfuric acid. The neutral polysaccharide consisted of acetylglucosamine and galactose, apparently in equimolar ratio. It is of considerable interest that the latter compound had previously been associated with the agglutinating factor of Group A blood. (Group II of the Moss system.)

From the vitreous humor and the umbilical cord, polysaccharide acids have been prepared which on hydrolysis yield the same products as are obtained from gastric mucin. However, the analytical data given by Meyer and Palmer for the various components (hexuronic acid, acetyl, nitrogen, glucosamine) indicate that the mucoitinsulfuric acid derived from gastric mucin may not be the same as the ones derived from the vitreous humor and the umbilical cord. The last two are apparently identical with the polysaccharide acid derived from certain strains of streptococci (Meyer, Dubos, and Smyth).

Glucosides. The glucosides are substances which on hydrolysis yield a sugar, usually glucose, and one or more additional products. The glucosides are very numerous and are widely distributed in plants. Familiar examples are *phlorhizin*, also spelled *phloridzin* (glucose + phloretin), which occurs in the bark of *Rosaceae*; *coniferin* (glucose + coniferyl alcohol), present in the bark of the fir tree; *salicin* (glucose + saligenin) in the bark of the willow tree; *amygdalin* (2 glucose + mandelonitrile), in the seeds of the bitter almond; *quercitrin* (rhamnose + quercetin), in the bark of dyer's oak; *sinigrin* (glucose + allyl thiocyanate + $KHSO_4$), in black mustard seeds; *hesperidin* (rhamnose + 2 glucose + hesperetin) in orange and lemon juice, Hungarian red pepper, etc. The digitalis glucosides found in the leaves of the foxglove are of special importance, owing to their powerful action on the heart. These include digitoxin, $C_{41}H_{64}O_{13}$ (digitoxigenin + 3 digitoxose, $C_6H_{12}O_4$); digitalin, $C_{36}H_{56}O_{14}$ (dianhydrogitoxigenin + glucose + digitalose, $C_7H_{14}O_5$); gitoxin, $C_{41}H_{64}O_{14}$ (gitoxigenin + 3 digitoxose). Equally interesting are the closely related glucosides of strophanthus, *k*-strophanthin-β, $C_{36}H_{54}O_{14}$ (strophanthidin + glucose + cymarose, $C_7H_{14}O_4$) and ouabain, $C_{29}H_{44}O_{12}$ (ouabagenin + rhamnose), and of the sea onion or squill, scillarin A, $C_{36}H_{52}O_{13}$ (scillaridin A + glucose + rhamnose).[61]

[60] K. Meyer and J. W. Palmer, *J. Biol. Chem.*, **114**, 689 (1936); K. Meyer, R. Dubos, and E. M. Smyth, *ibid.*, **118**, 71 (1937); Meyer, Smyth, and Palmer, *ibid.*, **119**, 73, 491, 501, 507 (1937).

[61] The "genins" or "aglucones" of these so-called cardiac glucosides belong to the phenanthrene group of compounds and are therefore related to the sterols (p. 78), bile acids, vitamins D, the sex hormones, and other compounds of physiological importance. For a comprehensive description of these compounds the reader is

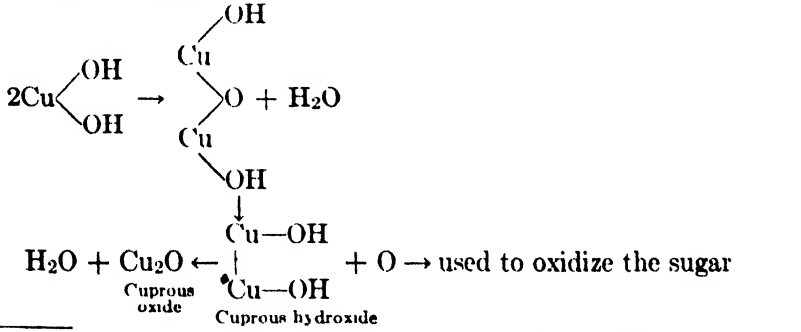
In the animal body compounds with a glucoside linkage are also found. The cerebrosides are usually classified with the lipids but may also be considered as galactosides. The two more familiar cerebrosides are phrenosin and kerasin. When hydrolyzed, these yield fatty acids, galactose, and sphingosine, a nitrogenous base. Then there are the nucleosides which give on hydrolysis a sugar, *d*-ribose, and either a purine or pyrimidine.

Pectins. Pectins are carbohydrates of high molecular weight and colloidal properties which occur most abundantly in the parenchymatous tissues of fruits and vegetables, such as apples, oranges, grapefruit (in the last two, especially in the inner white rind), turnips, and beets. On hydrolysis, the pectins yield galacturonic acid, arabinose, and galactose. In the presence of suitable concentrations of acid and sucrose, the pectins form the familiar fruit jellies and jams.[62]

REACTIONS OF THE SUGARS

Sugars that have an aldehyde or ketone group are easily oxidized, thereby acting as reducing agents. Of the many tests that are known, a few will be described here. For detailed directions, the student is referred to biochemical laboratory manuals.

Fehling's Test. Two solutions are used: one contains copper sulfate, the other Rochelle salt and sodium or potassium hydroxide. Two or three cubic centimeters of each solution are mixed and heated. At this stage, heating should produce no change. Upon the addition of several drops of sugar solution and further heating, the solution at first becomes turbid and greenish in color. Later a yellow precipitate of cuprous hydroxide or a red precipitate of cuprous oxide separates. The cupric hydroxide which forms when the two solutions are mixed is held in solution by the tartrate (Rochelle salt is sodium-potassium tartrate). The reduction of the copper hydroxide by the sugar may be considered to take place as follows:



referred to the monograph by L. F. Fieser, "Chemistry of Natural Products Related to Phenanthrene," New York, 1936.

[62] The pectins and the related classes of substances known as gums and mucilages are more adequately described in Gortner's "Outlines of Biochemistry," John Wiley & Sons, New York, 1938. Second Edition.

Fehling's test is mainly of historical interest and has been replaced by more sensitive tests.

Benedict's Test. Benedict's solution contains sodium carbonate, sodium citrate, and copper sulfate. After preliminary heating of the reagent, several drops of sugar solution are added. A greenish yellow or red precipitate is produced on heating a second time. Benedict's solution and test have been modified for use in the quantitative estimation of sugar. Here, however, the end-point is indicated by the disappearance of the blue color, owing to the formation of white cuprous thiocyanate (CuCNS).

Nylander-Almen Test. In this test, bismuth subnitrate is reduced by the sugar to metallic bismuth according to the following equation:

$$2Bi(OH)_2NO_3 + 2KOH \rightarrow 2Bi(OH)_3 + 2KNO_3$$

$$2Bi(OH)_3 \rightarrow Bi_2 + 3H_2O + 3O$$

Barfoed's Test. The addition of sugar solution to Barfoed's reagent (containing copper acetate in dilute acetic acid), with heating, results in the reduction of the cupric acetate to cuprous oxide. The test is given by the monosaccharides but not readily by the disaccharides; hence it is employed in distinguishing the two groups.

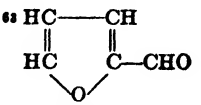
Molisch Test. This is a general test for all carbohydrates as well as for other compounds containing a carbohydrate residue in their molecules. Several drops of an alcoholic solution of α-naphthol are added to the sugar solution. This is then stratified above a layer of concentrated sulfuric acid. At the zone of contact, a violet ring develops. The reaction depends on the condensation of furfural [63] or its derivatives with the α-naphthol.

Seliwanoff Reaction. This test is specific for ketohexoses. Seliwanoff's reagent is a solution of resorcinol in hydrochloric acid. On heating, the acid converts fructose, for example, into levulinic acid and hydroxymethylfurfural. The latter compound condenses with the resorcinol to form a red compound.

Orcinol-Hydrochloric Acid Test. The addition of this reagent to a solution of a pentose, with heating, results in the production of a succession of colors—violet, blue, red, and green. If the sugar solution is sufficiently concentrated, a bluish green precipitate separates.

Phloroglucinol-Hydrochloric Acid Test. The addition of phloroglucinol and hydrochloric acid to a pentose solution, with heating, results in the development of a cherry-red color. Galactose, likewise, responds to this test.

[63]



Furfural

Phenylhydrazine Reaction. The addition of phenylhydrazine to a solution of certain sugars (those having a free aldehyde or ketone group), with heating, results in the formation of yellow crystalline osazones, specific as to crystal form, melting-point, etc. By this method it is possible to distinguish between glucose, lactose, maltose, and other sugars. Glucose, fructose, and mannose yield the same osazone. Methylphenylhydrazine distinguishes glucose and fructose. Sucrose does not form an osazone.

Mucic Acid Test. Oxidation of galactose with hot nitric acid yields an insoluble dicarboxylic acid, mucic acid [COOH—$(CHOH)_4$—COOH]. The corresponding dicarboxylic acid formed from glucose, namely saccharic acid, is soluble. Lactose likewise yields mucic acid, since on hydrolysis it forms both glucose and galactose.

Fermentation. Yeast ferments glucose, fructose, maltose, sucrose, and other sugars with the formation of alcohol and carbon dioxide. Ordinary brewer's yeast (*Saccharomyces cerevisiae*) does not ferment either galactose or lactose. The disaccharides are first inverted by enzymes present in yeast.

Iodine Test. Iodine yields with starch a blue or purple-blue color. With glycogen and the higher dextrins a wine-red color is produced.

CHAPTER III

THE FATS AND RELATED COMPOUNDS

Fats are the triglyceride esters of fatty acids and are closely associated in nature with the phosphatides, cerebrosides, sterols, and other substances. There has been little uniformity in the nomenclature applied to these compounds. The name "lipoids" is often employed as an inclusive term, but it is also used in the more restricted sense of applying only to phosphatides and cerebrosides. Recently the term "lipides" or "lipids" has found a certain amount of favor as a general group name for the fats and fatlike substances.

The following classification has been suggested by Bloor: [1]

Lipids. Substances having the following characteristics:

a. Insolubility in water and solubility in the fat solvents, such as ether, chloroform, benzene.[2]

b. Relationship to the fatty acids as esters, either actual or potential.[3]

c. Utilization by living organisms.

Simple Lipids. Esters of the fatty acids with various alcohols.

1. Fats—esters of the fatty acids with glycerol.
2. Waxes—esters of the fatty acids with alcohols other than glycerol.

Compound Lipids. Esters of the fatty acids containing groups in addition to an alcohol and fatty acid.

1. Phospholipids—substituted fats containing phosphoric acid and nitrogen: lecithin, cephalin (kephalin), sphingomyelin.
2. Glycolipids—compounds of the fatty acids with a carbohydrate and containing nitrogen but no phosphoric acid: phrenosin, kerasin. These are also called cerebrosides.

[1] W. R. Bloor, "Biochemistry of Fats," *Chem. Rev.*, **2**, 243 (1925–6).

[2] This property, namely solubility in fat solvents and insolubility in water, sets off the fats from the carbohydrates and proteins. Nevertheless, this property is not an absolute one; the lecithins are somewhat soluble in water and insoluble in acetone which is otherwise a good solvent for fats. The cephalins are mainly insoluble in alcohol, while sphingomyelin and the cerebrosides are difficultly soluble in ether.

[3] Bloor has wisely included this property, as well as the next one (*c*), in order to exclude organic compounds which have no biochemical relationship to the fats or fatty acids, but which from their solubilities alone would be included in the group. According to (*b*) and (*c*), the substances classified as lipids must be either ester-like combinations of the fatty acids or capable of forming such combinations, and they must be capable of performing some useful functions in living organisms.

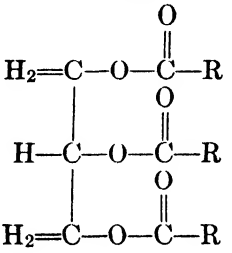
3. Aminolipids, sulfolipids, etc.—groups which are at present not sufficiently well characterized for classification.

Derived Lipids. Substances derived from the above groups by hydrolysis.

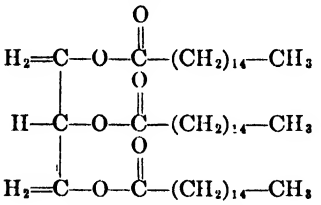
1. Fatty acids of various series.

2. Sterols—mostly alcohols of high molecular weight, found in nature combined with fatty acids, and soluble in fat solvents: cholesterol ($C_{27}H_{45}OH$), myricyl alcohol ($C_{30}H_{61}OH$), cetyl alcohol ($C_{16}H_{33}OH$), etc.

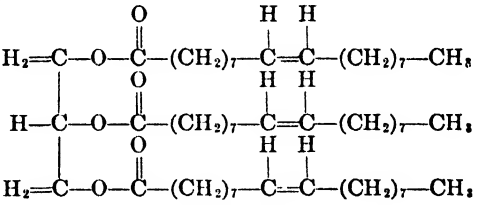
Constitution of the Fat Molecule. The molecular structure of a fat may be represented by the formula:



in which R represents a fatty acid chain. The following are the formulas for tripalmitin (the tripalmitic acid ester of glycerol) and triolein (the trioleic acid ester of glycerol):

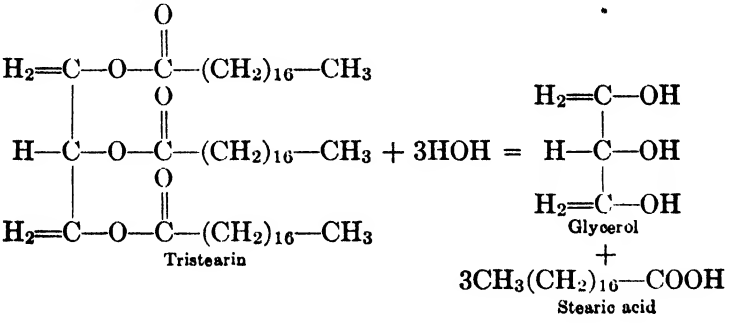


Tripalmitin or palmitin



Triolein or olein

Fats are hydrolyzed by the action of acids, alkalies, fat-splitting enzymes (lipases), and superheated steam. Three molecules of fatty acid and one of glycerol are formed as a result.



Tristearin

Glycerol

Stearic acid

In the presence of alkali, the fatty acid reacts to form soap. The action of alkali on fat is therefore termed saponification. Saponification, in a broader sense, is the hydrolysis of any ester with or without alkali.

Saturated Fatty Acids. Fatty acids may be divided into two general groups, the saturated and the unsaturated. With few exceptions, the fatty acids that occur in nature contain an even number of carbon atoms. However, in the blubber of porpoises is found isovaleric acid ($C_5H_{10}O_2$), and in croton oil, tiglic acid ($C_5H_8O_2$), which is an unsaturated fatty acid. The saturated fatty acids are homologues of formic acid and have the general formula $C_nH_{2n}O_2$, ($C_nH_{2n+1}COOH$).

Formic acid (HCOOH) occurs in sweat, urine, meat juice, and the bodies of ants (especially the red ant).

Acetic acid (CH_3COOH) occurs in vinegar; in smaller amounts in sweat, muscle and other tissues, feces, and urine. It occurs as a glyceride in the oil of the spindle tree.

Butyric acid, $CH_3 \cdot (CH_2)_2 \cdot COOH$, is present as a glyceride in butter, to the extent of about 6 per cent. The free fatty acid occurs in sweat.

n-Caproic acid, $CH_3 \cdot (CH_2)_4 \cdot COOH$, and *n*-caprylic acid, $CH_3(CH_2)_6 \cdot COOH$, occur as glycerides in butter, coconut oil, and palm-nut oil.

Capric acid, $CH_3 \cdot (CH_2)_8 \cdot COOH$, is present, in combination with glycerol, in the milk of cows and goats, as well as in coconut oil and palm-nut oil.

Lauric acid, $CH_3 \cdot (CH_2)_{10} \cdot COOH$, occurs as glyceride in milk, more abundantly in spermaceti, laurel oil, coconut oil, palm-kernel oil, etc.

Myristic acid, $CH_3 \cdot (CH_2)_{12} \cdot COOH$, is a constituent of nutmeg oil and also occurs as glyceride in milk and vegetable fats. In small amounts it has been found in lard and cod-liver oil.

Palmitic acid, $CH_3 \cdot (CH_2)_{14} \cdot COOH$, is widely distributed as glyceride in animal and vegetable fats. It occurs in cow's milk, myrtle wax, Japan wax, and palm oil. Bayberry tallow is almost pure tripalmitin. In spermaceti, a wax found in the skulls of whales and dolphins, it is present in combination as the ester of cetyl alcohol ($C_{16}H_{33}OH$); in beeswax as the ester of myricyl alcohol ($C_{30}H_{61}OH$ or $C_{31}H_{63}OH$); and in opium wax as the ester of ceryl alcohol ($C_{26}H_{53}OH$).

Stearic acid, $CH_3 \cdot CH_2)_{16} \cdot COOH$, is contained as a glyceride in most vegetable and animal fats.

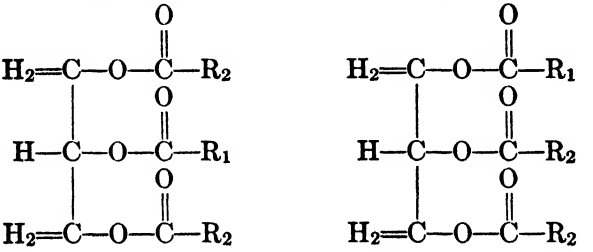
Arachidic acid, $CH_3 \cdot (CH_2)_{18} \cdot COOH$, occurs in peanut and other vegetable oils. It is also said to be present in cow's milk, and in the fat of tissues and of dermoid cysts.

There are a number of fatty acids of even greater complexity. Behenic acid, $CH_3(CH_2)_{20} \cdot COOH$, is found in the oil of ben obtained from the seeds of *Moringa pterygosperma*. It is a constituent of butterfat (Bosworth and Sisson).[4] Lignoceric acid, $CH_3(CH_2)_{22} \cdot COOH$, is a component of the phosphatide, sphingomyelin, and occurs also in

[4] *J. Biol. Chem.*, **107**, 489 (1934).

beechwood and lignite tar. It is present as the glyceride in peanut oil, and, in small amounts, in butter. Cerotic acid ($C_{26}H_{52}O_2$) has been isolated from a variety of waxes (beeswax, carnauba wax, Chinese wax, opium wax, and wool fat). Helz and Bosworth [5] have recently shown it to be present in butterfat. Melissic acid ($C_{30}H_{60}O_2$) occurs free in beeswax.

Mixed Triglycerides. The three fatty-acid radicals in a fat molecule may be all the same, as in palmitin, stearin, and olein; but it is also possible for them to differ. Thus, if the three fatty acids are all different, three combinations are possible, each representing a different mixed triglyceride. If two radicals are alike and one is different, two combinations of these radicals are possible, namely:



Other combinations may exist. Taking into consideration the number of fatty acids occurring naturally, it is theoretically possible to have innumerable mixed triglycerides. A number of these have been isolated. Palmito-distearin occurs in lard and in beef tallow. Stearo-dipalmitin has been prepared from mutton tallow. Stearo-diolein is said to be present in the fat of the human body. Myristo-palmito-olein occurs in cacao-butter.

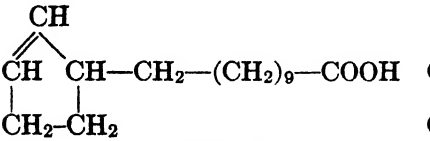
Unsaturated Fatty Acids. The unsaturated fatty acids contain one or more pairs of carbon atoms united by a double bond. In the oleic series of fatty acids ($C_nH_{2n-2}O_2$ or $C_nH_{2n-1}COOH$) there is one such pair. Tiglic acid, $C_5H_8O_2$, occurs in croton oil. Several isomers of the formula $C_{16}H_{30}O_2$ have been described. Hypogeic acid occurs in peanut and maize oils. Palmitoleic acid is said to be present in cod-liver oil. Physetoleic acid occurs in sperm and seal oils. The most important member of the series is oleic acid, $C_{18}H_{34}O_2$, $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$, a constituent of most fats and oils, where it is present in combination with glycerol. A number of fatty acids isomeric with oleic acid have been prepared in the laboratory (elaidic and isoöleic acids). Another isomer, rapic acid, is present as glyceride in rape or colza oil. Gadoleic acid ($C_{20}H_{38}O_2$) occurs in herring, sperm, and cod-liver oils. Erucic acid ($C_{22}H_{42}O_2$) has been found in rape-seed, mustard-seed, and cod-liver oils.

Belonging to the linoleic or linolic acid series ($C_nH_{2n-4}O_2$) and the linolenic acid series ($C_nH_{2n-6}O_2$) are fatty acids of a greater

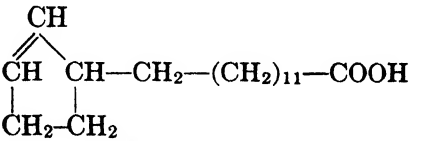
[5] *Ibid.*, **116**, 203 (1936).

degree of unsaturation than oleic acid. Linoleic or linolic acid, $C_{18}H_{32}O_2$, $CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$, is an important constituent of cottonseed oil, and linolenic acid, $C_{18}H_{30}O_2$, $CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$, of linseed oil. When exposed to the air, the highly unsaturated triglycerides of linolenic acid combine readily with oxygen to form solid compounds. To this are due the useful properties of linseed and other drying oils. Clupanodonic acid ($C_{22}H_{34}O_2$) has been prepared from cod-liver and sunfish-liver oils and from herring, sardine, and whale oils. Castor oil contains a monohydroxy fatty acid, ricinoleic acid ($C_{18}H_{34}O_3$), and a dihydroxy acid, dihydroxystearic acid ($C_{18}H_{36}O_4$). Arachidonic acid ($C_{20}H_{32}O_2$) has been isolated from liver tissue of pigs and is reported to be the only highly unsaturated fatty acid occurring in thyroid, suprarenal and spleen. Arachidonic acid, and possibly tetracosapentenoic acid, $C_{24}H_{38}O_2$, are said to occur in the brain, where the highly unsaturated fatty acids seem to be present in greater proportion than in other tissues (Brown).[6] Bosworth and Brown have reported the presence in butterfat of the following unsaturated fatty acids: decenoic ($C_{10}H_{18}O_2$), tetradecenoic ($C_{14}H_{26}O_2$), and arachidonic acid ($C_{20}H_{32}O_2$). The occurrence of the higher saturated fatty acids—lignoceric, behenic, and cerotic —in glyceride linkage in butter has been mentioned.

Two cyclic unsaturated fatty acids are of importance because of their therapeutic value in the treatment of leprosy. Both are found in chaulmoogra oil. They are hydnocarpic acid ($C_{16}H_{28}O_2$) and chaulmoogric acid ($C_{18}H_{32}O_2$).



Hydnocarpic acid



Chaulmoogric acid

Properties of Fats and Fatty Acids. *Solubility.* Mention has been made of the solubility of fats in the so-called fat solvents, ether, chloroform, and benzene, and their insolubility in water. This applies more especially to the glycerides of the higher fatty acids, for those of the lower fatty acids, such as tributyrin and tricaproin, are somewhat soluble in water. In ethyl and methyl alcohol and in acetone, the fats dissolve readily in the hot, but only slightly in the cold.

All fatty acids are soluble in ether, chloroform, benzene, and hot alcohol. The fatty acids, lower than palmitic acid, are also soluble in cold alcohol. In water only the lowest members are readily soluble.

Hydroxy fatty acids, as well as their glycerides, are insoluble in petroleum ether, a property which distinguishes them from other fatty acids and fats.

[6] *J. Biol. Chem.*, **83**, 777, 783 (1929); **89**, 167 (1930); A. W. Bosworth and J. B. Brown, *ibid.*, **103**, 115 (1933).

The fats themselves are very good solvents for other fats and fatty acids.

Consistency, Melting-point, Solidification-point. The temperature at which a fat melts is higher than the temperature at which it solidifies. Thus, the melting-point of tristearin is 71.5° C., whereas the solidfying-point is 52.5° C. This peculiarity of having widely differing solidifying- and melting-points is not exhibited by the fatty acids. The melting-point of a fat depends upon the component fatty acids. The glycerides of the higher saturated fatty acids have higher melting-points than the glycerides of the lower fatty acids. The glycerides of the unsaturated fatty acids have even lower melting-points. Glycerides which are fluid at ordinary temperatures are commonly called oils; those which are solid are called fats. This distinction, which is essentially one of convenience in industrial and culinary uses, is not ordinarily adhered to in chemical discussions, the term fat being just as appropriate for a liquid as for a solid fat.

Specific Gravity. The specific gravity of most solid fats (mutton tallow, lard, beef tallow, coconut butter, etc.) is very uniform, being approximately 0.86. Somewhat greater variation is found with different liquid fats, as is shown by the following data: olive oil, 0.915–0.918; peanut oil, 0.917–0.926; cottonseed oil, 0.921–0.926; maize oil, 0.921–0.927; linseed oil, 0.931–0.941.

Other physical constants of value in the identification of fats and in determining their purity are the viscosity and refractive index.

Saponification Value. When treated with basic hydroxides, fats yield glycerol and the salts of fatty acids. The latter are termed soaps, and the process by which they are formed is called saponification. The soaps of commerce are usually those of sodium and potassium. Calcium and magnesium soaps are very insoluble in water.

$$\underset{\text{Stearin}}{C_3H_5(C_{18}H_{35}O_2)_3} + 3KOH = \underset{\text{Glycerol}}{C_3H_5(OH)_3} + \underset{\text{Potassium stearate (a soap)}}{3CH_3(CH_2)_{16}COOK}$$

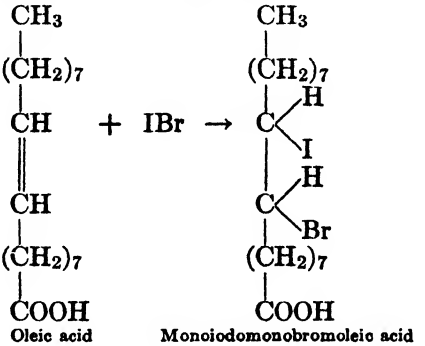
Applying the law of chemical combination to the above equation, we see that the molecular weight of fat is to three times the molecular weight of alkali as the actual weight of fat is to the actual weight of alkali. If the last three terms are known, the first can be determined. The amount of potassium or sodium hydroxide that will react with a given amount of fat in the process of saponification will depend on the average length of the constituent fatty-acid chains, for the smaller the fatty-acid molecules, the greater would be their number in a given amount of fat. Upon this principle is based a method for determining the character of different fats. The determination is made by heating a definite amount of fat (usually 1–2 grams) with a known volume of a standardized alcoholic solution of potassium hydroxide (usually 25 cc. of 0.5 normal alkali) until saponification is complete. The unused alkali is determined by titration with standard acid. From the data obtained may be cal-

culated the amount of alkali that was used in the saponification of the fat, and in turn its saponification value. *The saponification value is defined as the number of milligrams of potassium hydroxide neutralized by the saponification of one gram of fat.* Accordingly, it serves as a measure of the mean molecular weight of the fatty acids that are present in the fat. A large proportion of butter consists of the lower fatty acids; hence, butter has a relatively high saponification value (about 220–230). Similarly, coconut oil contains such large amounts of caproic, caprylic, capric, and lauric acid that it has an even higher saponification number (about 250). On the contrary, lard, mutton tallow, and cod-liver oil are composed of the higher fatty acids to a greater extent. Consequently, these have relatively low saponification values.

Most fats contain from 0.3 to 2.5 per cent of unsaponifiable matter, composed mainly of sterols. The body oil of the porpoise contains 16–17 per cent of unsaponifiable matter.

Hydrogenation. The unsaturated fats, such as those contained in vegetable oils, may be saturated by hydrogen, a reaction which is catalyzed by certain finely divided metals, including nickel. By the introduction of two hydrogen atoms at the unsaturated bond in oleic acid, stearic acid is formed, and similarly linoleic acid may be converted by hydrogenation, first to oleic and finally to stearic acid. This process is of great commercial and economic importance, as it makes possible the production of valuable articles of diet from comparatively inedible oils, such as cottonseed oil. The process of hydrogenation is not carried to completion, however, as this would produce a brittle form of tallow. The various lard substitutes of commerce are the products of partial hydrogen absorption and contain approximately 20 to 25 per cent of saturated fats (stearin), 65 to 75 per cent of olein, and 5 to 10 per cent of linolein. The hydrogenated fats are as well utilized by the animal body as the natural fats.

Halogen Absorption; Iodine Number. The unsaturated fatty acids react readily with the halogens, particularly with iodine chloride and iodine bromide, forming saturated halogen absorption derivatives.



Oleic acid — Monoiodomonobromoleic acid

The more unsaturated the fatty acid, the more halogen is taken up, the reaction being quantitative under certain conditions. Upon this principle is based a method for determining the degree of unsaturation of fats and fatty acids. In determining the iodine number, a weighed amount of fat is treated with a known volume (usually 25 cc.) of a solution containing iodine chloride or iodine bromide (Wijs or Hanus solution) and allowed to stand in the dark for one to two hours. The unabsorbed halogen is then determined by titration with standard sodium thiosulfate. From the data thus obtained, the amount taken up by the fat may be calculated in terms of iodine, from which the iodine number may be computed. *The iodine number is the number of grams of iodine that is absorbed by 100 grams of fat.* No halogen is taken up by saturated fatty acids or their corresponding glycerides. Oleic acid has one double bond and an iodine value of 90.1. Clupanodonic acid has five double bonds and an iodine number of 384. The iodine number therefore serves as an index of the degree of unsaturation of fats.

Additional Criteria. Certain other analytical procedures provide additional criteria for the identification of fats and the determination of their purity. *The acid number* is the number of milligrams of KOH required to neutralize the free fatty acid in 1 gram of fat. It is determined by titration of a weighed sample of fat, dissolved in hot, neutral alcohol, with standard alkali solution. Rancidity in fat, owing to the presence of free fatty acid in relatively large amount, is indicated by a high acid number.

The *acetyl value* is a measure of the number of OH groups present in a fat or oil. It is defined as the number of milligrams of KOH required to neutralize the acetic acid derived from the saponification of 1 gram of acetylated fat. The method of analysis requires the preliminary acetylation of the fat with acetic anhydride, followed by saponification of a weighed amount of the acetylated fat with alcoholic KOH, liberation of all the fatty acid with a calculated amount of mineral acid, separation of the acetic acid from insoluble fatty acids and its titration with standard alkali. Castor oil, owing to its high content of ricinoleic acid, has a high acetyl value (146–150.5); for most other fats the values vary from 1 to 20.

A measure of the volatile soluble fatty acids is given by the *Reichert-Meissl number.* It is defined as the number of cubic centimeters of 0.1 *N* alkali required to neutralize the volatile, soluble fatty acids distilled from 5 grams of saponified and acidified fat. The *Polenske number* is a measure of the insoluble, volatile fatty acids.

On page 72 are given certain of the physical and chemical constants of a number of the more common fats and oils.

Rancidity and Oxidation of Fats. Fats are relatively unstable substances and are susceptible to deterioration, especially when exposed to light, heat, and moisture. They thus acquire characteristically disagreeable odors and flavors. At least two changes occur, one consisting in

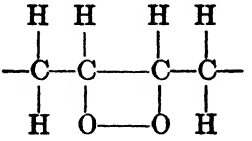
TABLE X

CONSTANTS OF FATS AND OILS *

Common Name	Specific Gravity at 15° C. / 15° C.	Refractive Index at 25° C.	Solidification Point ° C.	Saponification Number	Acid Number	Acetyl Number	Iodine Number	Reichert-Meissl Number	Unsaponifiable Matter
1. Beef tallow	0.895	... .	31 to 29	196–200	0.25	2 7–8 6	35 4–42.3	.....	....
2. Butterfat	0.907–0.912 $^{40°}_{15°}$	1.4555–1.4578$^{40°}$	19 to 24.5	210–230	0 45–35.4	1.9–8.6	26–28	17.0–34.5	0.3–0.45
3. Castor oil	0.950–0.967	1.4771	−12	175–183	0.12–0.8	146–150.5	84	1.4	0.6
4. Coconut oil	0.926	1.4537–1.4580$^{40°}$	14 to 22	253.4–262	1.1–1.9	1 97	6.2–10	6.6–7.5	0.2
5. Cod-liver oil	0.922–0.931	1.4758–1.4783	− 3	171–189	5.6	1 15	137–166	0.2	0.54–2.68
6. Corn oil	0.921–0.928	1.4733	−10 to −20	187–193	1.37–2.02	7.5–11.5	111–128	4.3	1.5–2.8
7. Cottonseed oil	0.917–0.918 $^{25°}_{25°}$	1.4743–1.4752	+12 to −13	194–196	0.6–0.9	21–25	103–111.3	0.95	1.1
8. Human fat	0.9179	1.459–1.4613$^{40°}$	15	193.3–199	.....	.....	64	0.25–0.55	....
9. Lard oil (fatty tissue)	0.934–0.938	1.4609–1.4620	27.1 to 29.9	195–203	0.5–0.8	2.6	47–66.5	.....	....
10. Linseed oil	0.930–0.938	1.4797–1.4802	−19 to −27	188–195	1–3 5	.....	175–202	0.95	0.4–1.2
11. Olive oil	0.915–0.920	1.4657–1.4667	2	185–196	0 3–1.0	10.5	79–88	0.6–1.5	0.4–1.0
12. Palm oil	0.924	1.4603–1.4639$^{40°}$	..... .	200–205	10	15.7	49.2–58.9	0.9–1.9	....
13. Peanut oil	0.917–0.926	1.4620–1.4653$^{40°}$	3	186–194	0 8	3.5	88–98	0.4	0.5–0.9
14. Sesame oil	0.919 $^{25°}_{25°}$	1.4704–1.4717	−4 to −16	188–193	9.8	.....	103–117	1.1–1.2	0.95–1.32
15. Wool fat	0.970–0.973	. .	.. .. .	82–130	59.8	23	17–29	8	39–44

* Taken from "Handbook of Chemistry and Physics" by C. D. Hodgman and N. A Lange. *Courtesy Chemical Rubber Co.*

hydrolysis, with the liberation of free fatty acids. Rancid butter owes its peculiar odor partly to the formation of free butyric acid. The other change is one of oxidation and affects principally the unsaturated fatty acids, resulting in the fixing of oxygen in the peroxide form



as well as in the formation of aldehydes, ketones, and acids of low molecular weight. The two processes occur simultaneously, the presence of free acid apparently increasing greatly the susceptibility of fats to oxidation. The iodine number falls as rancidity progresses.

Oxidative rancidity with a predominance of ketone production has been attributed to certain microörganisms, notably fungi (*Aspergillus niger* and *Penicillium glaucum*). It is believed that the characteristic odor and flavor of Roquefort cheese are due largely to the production of methyl ketones and not to the esters of butyric acid.

Oxidation of linolenic acid produces no very sharp off odors. Somewhat more definite off odors are obtained when linoleic acid is oxidized, and, in the case of oleic acid, these are very marked. Heptylic and nonylic aldehydes are among the odoriferous and rancid-tasting products resulting from the oxidation of oleic acid.

The capacity to take up oxygen is especially conspicuous in the highly unsaturated fats and is a property characteristic of the so-called drying oils. When thin layers of these oils are exposed to air, they absorb oxygen and are converted into tough, elastic, and waterproof substances which adhere tightly to the painted surface and protect it from the weather. Linseed oil, tung, or Chinawood oil, and soybean oil are the three principal drying oils used in the manufacture of paints, varnishes, artificial rubber, linoleum, and other coverings.[7]

Certain substances, such as catechol, hydroquinone, hydroxyhydroquinone, pyrogallol, and α-naphthol, inhibit the autoöxidation of fats. Antioxidants acting in a similar way are present in the unsaponifiable fraction of the natural fats and oils. These have been termed "inhibitols" by Olcott and Mattill,[8] whose researches on the subject are of outstanding importance. Inhibitols or antioxidants occur in wheat germ, cottonseed, corn, peanut, soybean, and other vegetable oils; in spinach, alfalfa, carrots, tomatoes, and lettuce. From the last of these an antioxidant has been isolated in crystalline form. In their physical and chemical

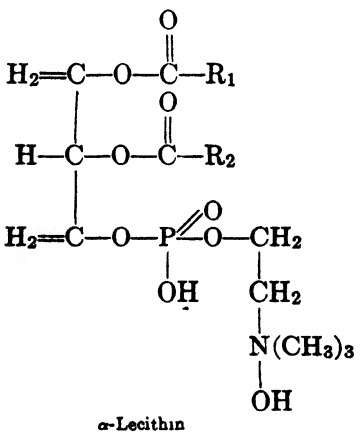
[7] For a general view of the economic importance of fats, the student is referred to the monograph by C. L. Alsberg and A. E. Taylor, "The Fats and Oils," Stanford University Press, 1928. For a more detailed description of the action of the drying oils the reader is referred to the book by H. B. Bull, "The Biochemistry of the Lipids," John Wiley & Sons, New York, 1937.

[8] *J. Am. Chem. Soc.*, **58**, 1627, 2204 (1936).

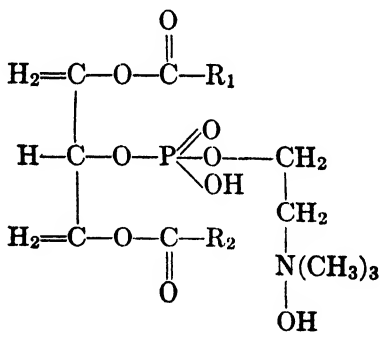
properties, the inhibitols of wheat germ and cottonseed oils, closely resemble vitamin E (p. 615).

Phospholipids. The phospholipids (also called phospholipins and phosphatides) are present in every animal and vegetable cell and are especially abundant in brain, heart, muscle, kidney, bone marrow, liver, and eggs. On hydrolysis these substances yield fatty acids, a nitrogenous base, phosphoric acid, and usually glycerol. Of the phospholipids that have been described, only three type substances have been studied sufficiently to establish their chemical individuality. These are the monoaminomonophosphatides, lecithin and cephalin; and the diaminomonophosphatide, sphingomyelin.

Lecithin. Hydrolysis of lecithin yields: (1) glycerophosphoric acid; (2) fatty acid; and (3) choline, $[(CH_3)_3{\equiv}N(OH){-}CH_2CH_2OH]$, trimethyloxyethyl ammonium hydroxide. Glycerophosphoric acid may be further hydrolyzed, the products being glycerol and phosphoric acid. There are two glycerophosphoric acids, the α- and β-forms, depending on the position of the ester linkage. Corresponding to these two forms there are two varieties of lecithin, as shown by the following formulas in which R_1 and R_2 represent fatty acid chains.



α-Lecithin



β-Lecithin

Egg yolk, liver, and brain are among the more abundant sources of lecithin. It has been reported [9] that α-lecithin predominates in brain and β-lecithin in egg yolk, whereas liver lecithin is composed of about equal parts of the α- and β-forms.

Lecithins have been described in which both of the constituent fatty acids are saturated. There are others in which both acids are unsaturated, and finally those containing both a saturated and an unsaturated fatty acid. From egg yolk have been isolated a saturated lecithin and one in which the constituent fatty acids are oleic and arachidonic.[10]

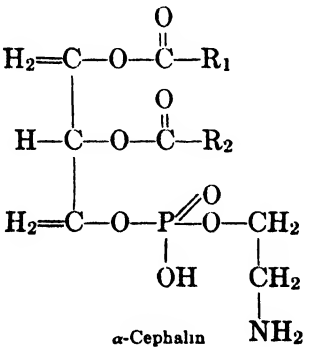
[9] J. J. Rae, *Biochem. J.*, **28**, 152 (1934).

[10] References to recent work on the fatty-acid components of lecithin are given by E. Klenk and K. Schuwirth, *Ann. Rev. Biochem.*, **6**, 115 (1937).

In a study of beef liver lecithin, Snider and Bloor [11] found the liquid (unsaturated) and solid (saturated) fatty acids to be present in the proportion of 55 : 40. Of the unsaturated acids, linoleic acid composed 45 per cent, arachidonic 31 per cent, and oleic 21 per cent. In small amounts, acids of larger molecular weight and of even greater unsaturation probably exist. Indeed, a C_{22} acid has been isolated from liver lipds which contains five ethylene bonds. It is dicosanpentenoic acid, $C_{22}H_{34}O_2$. On complete hydrogenation it is converted into behenic acid.

Brain lecithin also contains arachidonic as well as a highly unsaturated C_{22} acid. Brown [12] has reported the presence of tetracosanpentenoic acid, $C_{24}H_{38}O_2$, in beef and sheep brains, but its absence, except for traces, in hog's brain. In some of these studies, mixtures of phosphatides were employed, and there is therefore no certainty that these acids are actually components of lecithin, although this seems probable.

Cephalin. The methods in use for the separation of cephalin from lecithin and other associated substances are based principally on differences in solubility. The cephalin fraction may be precipitated by alcohol, in which it is somewhat less soluble than lecithin. Like lecithin, cephalin is widely distributed in animal tissues; it exists in two forms, α- and β-, and, at least theoretically, may hold in combination a variety of fatty acids. Stearic, oleic, and dicosantetranoic acids have been isolated. The occurrence of arachidonic acid has also been reported, but if present, the amount is very small. In lecithin, it will be recalled, arachidonic acid is an important component. The presence of a highly unsaturated C_{22} acid in brain cephalin has been reported. In addition to fatty acid, cephalin yields on hydrolysis glycerol, phosphoric acid, and a nitrogenous base, aminoethyl alcohol, or cholamin. α-Cephalin is represented by the following formula:



α-Cephalin

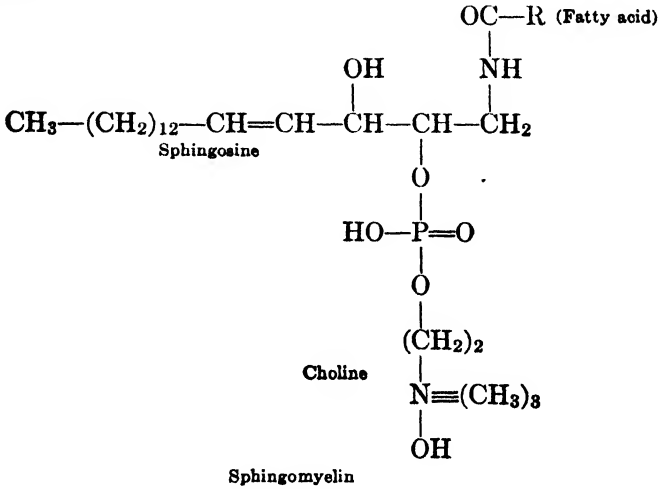
[11] *J. Biol. Chem.*, **99**, 555 (1932–33). Compare with the results obtained by R. G. Sinclair, *ibid.*, **111**, 261 (1935).

[12] *Ibid*, **83**, 783 (1929); J. B. Brown and W. C. Ault, *ibid.*, **89**, 167 (1930).

Properties of Cephalin and Lecithin. Lecithins and cephalins are miscible with water, from which they may be precipitated by acetone. Lecithin is soluble in alcohol, whereas cephalin is relatively insoluble. Both are soluble in ether, chloroform, benzene, and other common fat solvents, with the exception of acetone, in which they are insoluble. Lecithin and cephalin exhibit optical activity. The known lecithins are dextrorotatory. They oxidize readily in air, turning brown and acquiring a disagreeable odor.

It is believed that the formation of lecithin represents an intermediate stage in the metabolism of fatty acids. The neutral fat is presumably converted into lecithin, in which form the constituent fatty acids undergo a successive number of dehydrogenations, becoming more and more unsaturated. Lecithin is doubtless essential to the life of the cell, and even in extreme emaciation its content in cellular tissues remains unchanged. Preparations of lecithin obtained from tissues do not ordinarily represent a single substance, but, as has been pointed out, are actually mixtures of lecithins and may contain as impurities other phosphatides, as well as cerebrosides.

Sphingomyelin is a phospholipid, occurring in brain, kidney, liver, and egg yolk, which yields on hydrolysis phosphoric acid, two nitrogenous bases, choline and sphingosine, $[CH_3 \cdot (CH_2)_{12} \cdot CH{=}CH \cdot CH \cdot (OH) \cdot CH(OH) \cdot CH_2(NH_2)]$, and a fatty acid. Three fatty acids have been associated with sphingomyelin, namely, lignoceric acid $(CH_3 \cdot (CH_2)_{22} \cdot COOH$, stearic acid, and nervonic acid $[CH_3 \cdot (CH_2)_7 \cdot CH{=}CH \cdot (CH_2)_{13} \cdot COOH]$. Accordingly, there are at least three sphingomyelins, all of which may and apparently do occur in the same sphingomyelin fraction.



Sphingomyelin

Properties. Sphingomyelin is soluble in cold and hot chloroform, benzene, pyridine, glacial acetic acid, and hot alcohol, from which it

separates on cooling in crystalline form. It is relatively insoluble in hot and cold ether. It is somewhat soluble in hot acetone, but not in cold acetone, which may be used in precipitating it from water, in which it forms an opalescent suspension. Sphingomyelin is dextrorotatory. As compared with lecithin and cephalin, it is a relatively stable compound, undergoing no change on exposure to air or light.[13]

The Cerebrosides. Associated with phospholipids in the tissues, particularly in brain, are the cerebrosides which on hydrolysis yield the sugar galactose, the nitrogenous base sphingosine, and a fatty acid. The cerebrosides are glycolipids, and because galactose is the constituent sugar, they are also called *galactolipids* (or *galactolipines*). These compounds have a glucoside linkage.

Two cerebrosides have been known for a long time, *kerasin* and *phrenosin*. The fatty acid of kerasin is lignoceric acid The fatty acid of phrenosin is cerebronic acid, the constitution of which has long been a subject of controversy between Klenk[14] and Levene.[15] According to Klenk, cerebronic acid is α-hydroxylignoceric acid, $CH_3(CH_2)_{21}\cdot CHOH\cdot COOH$. On the other hand, Levene and fellow-workers contend that any one of several closely related hydroxy fatty acids (C_{23}, C_{24}, C_{25}) may enter into the constitution of phrenosin.

A third cerebroside, called *nervone*, was isolated by Klenk[16] in 1925. The fatty acid of this cerebroside is nervonic acid, $CH_3\cdot(CH_2)_7\cdot CH:CH\cdot(CH_2)_{12}CH_2\cdot COOH$. Its α-hydroxy derivative has been found among the hydrolytic products of a cerebroside mixture, from which the existence of a fourth cerebroside, *hydroxynervone* (*oxynervone*), has been assumed, although its actual isolation has not been accomplished.

The cerebrosides are soluble in hot alcohol, acetone, benzene, and pyridine. Like sphingomyelin, they are almost insoluble in hot and cold ether. The cerebrosides are optically active, phrenosin being dextrororatory, while kerasin and nervone are levorotatory.

Owing to the difficulty involved in isolating and purifying the various phosphatides and cerebrosides, mixtures of these substances have frequently been mistaken for individual compounds.

Waxes. Chemically defined, waxes are fatty-acid esters of monoatomic alcohols of high molecular weight, but ordinarily the term is applied loosely to a large variety of natural products, including certain secretions of insects and plants.

Beeswax is a familiar example. It is a mixture of substances, an

[13] An abnormal accumulation of sphingomyelin occurs in the brain, liver, and spleen in Niemann-Pick's disease.

[14] E. Klenk and R. Härle, *Z. physiol. Chem.*, **189**, 243 (1930); Klenk, *J. Biol. Chem.*, **105**, 467 (1934).

[15] F. A. Taylor and P. A. Levene, *J. Biol. Chem.*, **84**, 23 (1929); Levene and K. Heymann, *ibid.*, **102**, 1 (1933); Levene and P. S. Yang, *ibid.*, **102**, 54 1(1933).

[16] *Z. physiol. Chem.*, **145**, 244 (1925).

important constituent being the palmitic acid ester of myricyl alcohol ($C_{30}H_{61}OH$). The wax contains tetracosanic acid ($C_{24}H_{48}OH$) and its higher, even-carbon, homologues (C_{26} to C_{34}), as well as a series of primary alcohols (C_{24} to C_{36}). There also seem to be present saturated hydrocarbons of high molecular weight.

The wax of the outer covering, or cuticle, of the apple contains cerotic acid ($C_{26}H_{52}O_2$) and several of its higher, even-carbon homologues, as well as C_{26} to C_{30} primary alcohols and paraffin hydrocarbons. These are present, likewise, in Chinese grass wax and other waxes derived from leaf cuticles, such as carnauba wax, used in the manufacture of candles, shoe polish, varnish, etc.

Wool fat forms the coating over the surface of the fibers. It is actually a wax and contains among other substances the fatty-acid esters of cholesterol, agnosterol ($C_{30}H_{48}O$), and lanosterol ($C_{30}H_{50}O$). Spermaceti, found in the skull of certain species of whales and dolphins, contains as its chief constituent the palmitic acid ester of cetyl alcohol ($C_{16}H_{33}OH$). Among the other constituents of spermaceti are esters of lauric, myristic, and stearic acids with the following alcohols: lethol ($C_{12}H_{25}OH$), methol ($C_{14}H_{29}OH$), and stethol ($C_{18}H_{37}OH$).[17]

STEROLS

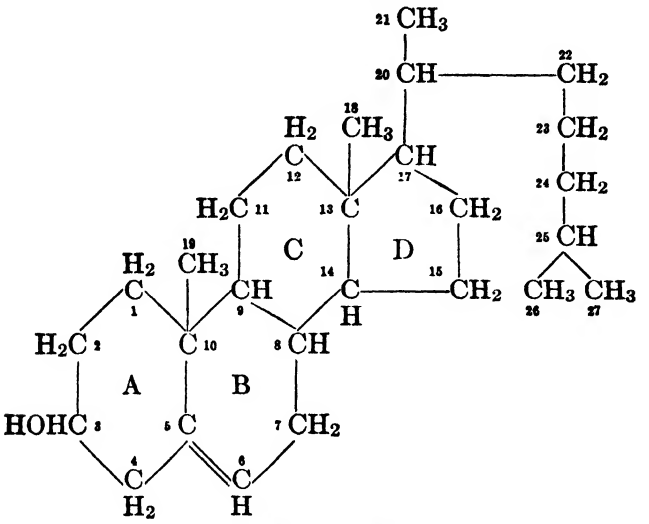
Cholesterol. The best known sterol is cholesterol ($C_{27}H_{45}OH$), first extracted from gallstones; hence the name (Gr. *chole*, bile, + *steros*, solid). Cholesterol is the principal sterol of the animal organism, and in association with other lipids is, in all probability, a constituent of every cell. It is also present in blood and bile. In the tissues and in blood serum it exists partly in combination with fatty acids, as cholesterol esters. Cholesterol is especially abundant in brain and nerve tissue, in the suprarenals, and in egg yolk. It is prepared commercially from the spinal cord of cattle.

Cholesterol has several asymmetric carbon atoms and is accordingly optically active $[\alpha]_D = -38.8$ (in chloroform solution). It is soluble in fat solvents, fatty acids (oleic), bile acids, and bile. It crystallizes from alcohol as white, glistening, rhombic plates with one irregular or notched corner. The melting-point is 150° C. Owing to the presence of an unsaturated bond, cholesterol combines with iodine and bromine. Hydrogenation at the double bond converts cholesterol into *cholestanol*. In common with other sterols, cholesterol yields a number of characteristic color reactions with certain reagents. In chloroform solution, the addition of acetic anhydride and sulfuric acid produces a blue (or violet-blue) color. This is known as the Leibermann-Burchard reaction; it is

[17] The constituents of plant waxes have been energetically studied in recent years by many workers, notably Chibnall (long series of papers in *Biochem. J.*, from 1930 on). As a result, our knowledge of these is far in advance of that of the waxes of insect and animal origin.

the basis of a quantitative method for the estimation of cholesterol in blood.

The chemical constitution of cholesterol, which seems to be definitely established, is represented by the following graphic formula:



Cholesterol ($C_{27}H_{45}OH$)

From this formula it is seen that the ring system is that of cyclopentenophenanthrene. Cholesterol is a secondary alcohol, the OH group occupying a definite position. The work that it took to determine the chemical constitution of cholesterol and related sterols was enormous and more or less paralleled similar investigations of the structure of the bile acids. Although the chemical relationship of these classes of compounds had long been suspected, their remarkable structural similarity was not realized or established until recently when some of the difficult problems were unraveled. It also became apparent that vitamin D, the sex hormones, the aglucones (aglycones) of the cardiac glucosides (p. 60), and other substances of physiological importance were likewise derivatives of phenanthrene, and indeed structurally related to the sterols and bile acids.[18]

[18] Callow and Young (*Proc. Roy. Soc.* [*London*], *A*, **157**, 194 [1936]) have proposed the term *steroid* for those compounds which are chemically related to cholesterol and contain a hydrogenated cyclopentenophenanthrene ring system. Under the term are included the sterols proper, the bile acids, cardiac aglycones, saponins, and sex hormones. This suggestion has been adopted by R. Schoenheimer and E. A. Evans, Jr., in their recent review, "The Chemistry of the Steroids," *Ann. Rev. Biochem.*, **6**, 139 (1937), to which the student is referred. It is also recommended that the student consult the review by O. Rosenheim and H. King, "The Chemistry of the Sterols, Bile Acids, and Other Cyclic Constituents of Natural Fats and Oils," *Ann. Rev. Biochem.*, **3**, 87 (1934). A comprehensive view of the chemistry of the sterols and related compounds will be found in L. Fieser's monograph, "The Chemistry of Natural Products Related to Phenanthrene."

From its widespread distribution in animal tissues and close chemical relation to other constituents of the body, it may be inferred that cholesterol is of unusual physiological importance. The maintenance of the normal permeability relations of cell membranes has been attributed partly to cholesterol. Cholesterol has also been described as the insulating medium of the myelin sheathes of nerves. Its abundance in brain and nerve tissue has been recognized for a long time. These and other considerations have been reviewed by Bills.[19] Considering existing information as a whole, it must be admitted that very little is actually known of the physiology of cholesterol.

Dehydrocholesterol and Other Sterols of Animal Origin. In the tissues, as well as in relatively pure preparations, cholesterol is accompanied by small amounts of other sterols. One of these is the unsaturated sterol, *7-dehydrocholesterol*, which on irradiation forms an antirachitic substance. The latter is identical with one of the naturally occurring vitamins D (p. 609).

Another derivative is the saturated sterol, *dihydrocholesterol*, $C_{27}H_{47}OH$. *Coprosterol* (Gr. *kopro*, dung) is an isomer of dihydrocholesterol. It is a constituent of feces and is presumably formed from cholesterol through the reducing action of bacteria in the lower bowel. Coprosterol has been prepared from cholesterol by hydrogenation.

Wool grease contains, in addition to cholesterol, *agnosterol* ($C_{30}H_{47}OH$) and *lanosterol* ($C_{30}H_{49}OH$). In the lower animals the widespread distribution of cholesterol has been known for a long time, but the coexistence of other sterols has been determined only recently. The sterol of the silkworm, *bombicysterol*, is, according to Bergmann,[20] a mixture composed of 85 per cent cholesterol and 15 per cent sitosterol, a plant sterol, or mixture of sterols. *Ostreasterol*, $C_{29}H_{47}OH$, occurs in oysters and clams.

Plant Sterols, or Phytosterols. The most widely distributed of the plant sterols is *sitosterol* (Gr. *sito*, grain), which once was considered a chemical entity, but which has been reported to consist of a mixture of at least three optical isomers, α, β, and γ (Anderson [21]). The separation of these isomers is exceedingly difficult, yet recently Wallis and Fernholz [22] described the fractionation of α-sitosterol from wheat germ oil into two compounds: one a sterol, of the formula $C_{30}H_{49}OH$; the other an isomer of stigmasterol, $C_{29}H_{47}OH$.

Stigmasterol was first isolated from the Calabar bean, where it occurs together with other sterols. It has also been found to be an important

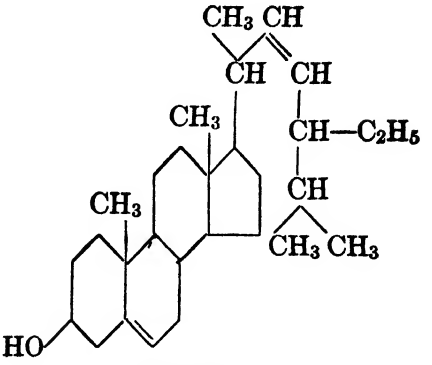
[19] C. E. Bills, "Physiology of the Sterols, Including Vitamin D," *Physiol. Rev.*, **15**, 1 (1935).

[20] *J. Biol. Chem.*, **107**, 527 (1934).

[21] R. J. Anderson and co-workers, *J. Am. Chem. Soc.*, **46**, 1450 (1924); **48**, 2972, 2976, 2987 (1926); *J. Biol. Chem.*, **71**, 389, 401 (1927).

[22] *J. Am. Chem. Soc.*, **58**, 2446 (1936).

constituent of soy bean oil. Its occurrence with other sterols in the oyster has been reported by Bergmann.[23]



Stigmasterol

Of considerable interest is ergosterol, $C_{28}H_{43}OH$, discovered by Tanret in ergot and later found to be present also in certain mushrooms and yeast; yeast is now the principal source of ergosterol. In 1927, it was discovered that ergosterol acquired the antirachitic property of vitamin D on irradiation. The active principle formed from ergosterol was isolated several years later, and for a time it seemed that this was in fact the naturally occurring vitamin, and that ergosterol was its provitamin. However, it is now realized that, although irradiated ergosterol (calciferol) possesses marked antirachitic potency, the vitamin D occurring in fish oils is related to other sterols (7-dehydrocholesterol, etc.). Further reference to these compounds will be made in a later chapter (p. 605).

In addition to ergosterol, yeast contains the unsaturated sterol, *zymosterol*, $C_{27}H_{43}OH$. Algae contain *fucosterol*, $C_{29}H_{47}OH$, while from cinchona bark a sterol of the formula $C_{29}H_{49}OH$ (*cinchol*) has been isolated.

[23] *J. Biol. Chem.*, 118, 499 (1937).

CHAPTER IV

THE PROTEINS

The resemblance between plant and animal proteins was recognized as early as 1839 by the Dutch chemist, Mulder,[1] who stressed the importance of these substances in the constitution of cell protoplasm. The proteins not only play an essential part in the regeneration and new formation of tissue, but also are used by the body in the production of energy. Most proteins contain at least the elements carbon, hydrogen, oxygen, nitrogen, and sulfur, and, as far as can be determined by chemical analysis, seem to be closely related. However, there is nothing more characteristic of the proteins as a group than their physiological specificity. No two proteins seem to be exactly alike in physiological behavior. The circulating and tissue proteins of one animal differ from those of all other animals, and the same is true of most plant proteins.

Proteins cannot be identified by the usual chemical methods, and, accordingly, the classification is based mainly on physical properties such as solubility. The classification that is most generally accepted, despite certain inconsistencies, was originally formulated and recommended by the American Physiological Society and the American Society of Biological Chemists.[2] This classification is given below:

THE PROTEINS

I. Simple Proteins. Protein substances that yield, on hydrolysis, only amino acids or their derivatives.[3]

[1] G. J. Mulder, *J. prakt. Chem.*, **16**, 129 (1889).

[2] *J. Biol. Chem.*, **4**, p. xlviii (1908).

[3] In the light of recent studies, this definition is not strictly correct. For example, egg albumin, serum albumin, globulin, and other proteins are known to contain carbohydrate groups. By definition, these should be classified as glycoproteins, a subgroup of the conjugated proteins, and not as simple proteins.

In a recent contribution, M. Sørensen and G. Haugaard [*Compt. rend. trav. lab. Carlsberg*, **19**, No. 12 (1933)] reported the presence of 1.71 per cent mannose in egg albumin recrystallized many times. An easily soluble serum ablumin, recrystallized several times, contained 0.47 per cent carbohydrate, consisting of equal amounts of mannose and galactose, and a sparingly soluble fraction containing 0.02 per cent carbohydrate. Horse serum globulin, several times precipitated, contained 1.82 per cent carbohydrate, likewise consisting of equal amounts of galactose and mannose. Casein contained 0.31 per cent galactose, but no lactose. Well-purified lactalbumin contained 0.44 per cent galactose, but no lactose. See also M. Sørensen, *ibid.*, **21**, *Ser. chim.*, 123 (1936).

The various simple proteins may be designated as follows:

a. Albumins. Simple proteins soluble in pure water and coagulable by heat. (Examples: egg albumin, serum albumin, legumelin of the pea, and leucosin of wheat.)

b. Globulins. Simple proteins insoluble in pure water but soluble in neutral solutions of salts of strong bases with strong acids. (Examples: serum globulin, fibrinogen, myosinogen of muscle, edestin of hemp seed, legumin of peas, excelsin in Brazil nuts, concanavalin in the jack bean.)

c. Glutelins. Simple proteins insoluble in all neutral solvents but readily soluble in very dilute acids and alkalies. (Examples: glutenin of wheat, oryzenin in rice.)

d. Alcohol-soluble proteins—Prolamins or Gliadins. Simple proteins soluble in relatively strong alcohol (70 to 80 per cent), but insoluble in water, absolute alcohol, and other neutral solvents. (Examples: gliadin from wheat or rye, hordein from barley, zein from maize or wheat.)

e. Albuminoids or Scleroproteins. Simple proteins that possess essentially the same chemical structure as the other proteins, but are characterized by great insolubility in all neutral solvents. These substances form the principal organic constituents of the skeletal structure of animals and also of their external covering and its appendages. (Examples: keratin from hair, horns, hoofs, nails, etc.; neurokeratin from gray substance of brain, spinal cord, and retina; elastin in elastic tissue, ligaments, and the walls of arteries; collagen in bones and cartilage; spongin found in the skeletal structure of the sponge; reticulin present in lung, kidney, spleen, liver, and lymphatic gland tissue; fibroin and sericin from silk.)

f. Histones. Soluble in water and insoluble in very dilute ammonia; in the absence of ammonium salts, insoluble even in an excess of ammonia. They yield precipitates with solutions of other proteins, and, on heating, a coagulum which is easily soluble in very dilute acids. On hydrolysis they yield a number of amino acids among which the basic ones predominate. (The histones are found in the red corpuscles of the blood and in spermatozoa. Examples: scombron in mackerel spermatozoa, gadus histone from the codfish, globin from hemoglobin.)

g. Protamins. Simpler polypeptides than the proteins included in the preceding groups. They are soluble in water, uncoagulable by heat, have the property of precipitating aqueous solutions of other proteins, possess strong basic properties, and form stable salts with strong mineral acids. They yield comparatively few amino acids, among which the basic amino acids greatly predominate. (Like the histones, the protamins occur in combination with nucleic acids in spermatozoa. Examples: salmine from salmon, sturine from sturgeon, scombrine from mackerel, cyprinine from carp, clupeine from herring.)

II. Conjugated Proteins. Substances that contain the protein molecule united to some other molecule or molecules otherwise than as a salt.

a. Nucleoproteins. Compounds of one or more protein molecules with nucleic acid. (Present in the germ of grain and in glandular tissue.)

b. Glycoproteins. Compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid. (Example: mucin.)[4]

c. Phosphoproteins. Compounds of the protein molecule with some as yet

[4] K. Meyer and J. W. Palmer have proposed the classification of glycoproteins into (1) mucins, (2) mucoids, (3) glucosidoproteins. The first group includes true mucins and sulfomucins, *Proc. Soc. Biol. Chem.*, *J. Biol. Chem.*, **114**, lxviii (1936).

undefined phosphorus-containing substance other than a nucleic acid or lecithin. (Examples: caseinogen of milk, vitellin of egg yolk.)[5]

d. Chromoproteins. Compounds of the protein molecule with hematin or some similar substance. (Examples: hemoglobin, hemocyanin.)

e. Lecithoproteins. Compounds of the protein molecule with lecithin. (Example: tissue fibrinogen.)

III. Derived Proteins.

1. *Primary Protein Derivatives.* Derivatives of the protein molecule, apparently formed through hydrolytic changes which involve only slight alterations of the protein molecule.

a. Proteans. Insoluble products which apparently result from the incipient action of water, very dilute acids, or enzymes.

b. Metaproteins. Products of the further action of acids and alkalies, whereby the molecule is so far altered as to form products soluble in very weak acids and alkalies but insoluble in neutral fluids. (Examples: acid metaprotein, alkali metaprotein.)

c. Coagulated Proteins. Insoluble products which result from (1) the action of heat on their solutions, or (2) the action of alcohols on the protein.

2. *Secondary Protein Derivatives.* Products of the further hydrolytic cleavage of the protein molecule.

a. Proteoses. Soluble in water, uncoagulated by heat, and precipitated by saturating their solutions with ammonium sulfate or zinc sulfate.

b. Peptones. Soluble in water, uncoagulated by heat, but not precipitated by saturating their solutions with ammonium sulfate.

c. Peptides. Definitely characterized combinations of two or more amino acids, the carboxyl group of one being united with the amino group of the other, with the elimination of a molecule of water.

In the classification adopted by British biochemists, the protamins, histones, albumins, globulins, glutelins, gliadins, scleroproteins, and phosphoproteins are grouped as the simple proteins. The conjugated proteins are the glucoproteins, nucleoproteins, and chromoproteins. The metaproteins or infraproteins, proteoses, and polypeptides are grouped as products of protein hydrolysis.

Amino Acids. Protein molecules are built up of hundreds, and even thousands, of amino acids. By the action of mineral acids or protein-splitting enzymes, they may be broken down into simpler and simpler compounds, the end-products being the amino acids. The first of these was discovered more than a century ago. Since then many amino acids have been described as cleavage products of the protein molecule, but even now only twenty-three are generally recognized as such. In a review dealing with the history of the discovery of the amino acids, Vickery and Schmidt[6] have suggested certain criteria as the basis for considering an amino acid as a definite product of protein hydrolysis. Before applying these criteria it is assumed that the substance in ques-

[5] The tendency now is to regard vitellin as a lecithoprotein, since it occurs together with large amounts of lecithin in the egg yolk and cannot be completely freed from lecithin except by rather drastic extraction (Gortner).

[6] *Chem. Rev.*, **9**, 169 (1931).

tion has been liberated by hydrolysis from a protein of demonstrated purity and has been adequately characterized by analysis of salts and typical derivatives. The essential criteria imposed by Vickery and Schmidt are:

(1) The amino acid must have been isolated by some worker other than the discoverer.

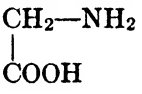
(2) Its constitution must have been established by synthesis and by demonstration of identity between the synthetic product and the racemized natural product, or by actual resolution of the synthetic product and preparation of the optically active natural isomer.

CLASSIFICATION OF AMINO ACIDS

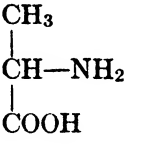
I. Aliphatic Amino Acids.

A. Monoaminomonocarboxylic Acids.

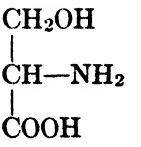
1. Glycine, $C_2H_5NO_2$, or aminoacetic acid. (1820, Braconnot.[7])



2. *d*-Alanine, $C_3H_7NO_2$, or α-aminopropionic acid. (Weyl, 1888.)

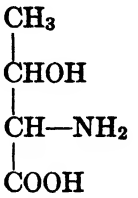


3. *l*-Serine, $C_3H_7NO_3$, or α-amino-β-hydroxypropionic acid. (1865, Cramer.)

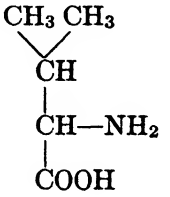


[7] The dates of discovery of these amino acids as *products of hydrolysis of proteins* and the names of the discoverers are those given by H. B. Vickery and C. L. A. Schmidt, *Chem. Rev.*, **9**, 169–318 (1931). Wherever possible, these have been confirmed by reference to original sources. It is to be pointed out that not in all cases were the constitutions of the amino acids known to their discoverers, nor are the names now in use necessarily those originally given. To cite a recent example as an illustration, methionine was discovered by Mueller, but its constitution was determined by Barger and Coyne (*Biochem. J.*, **22**, 1417 [1928]), who suggested the name. Then, also, at least two amino acids, alanine (Streiker, 1850) and proline, were synthesized long before they were obtained as decomposition products of protein.

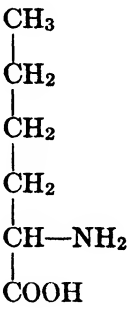
4. *d*-Threonine, $C_4H_9NO_3$, or α-amino-β-hydroxybutyric acid. (1925, Schryver and Buston; 1935, McCoy, Meyer and Rose.)



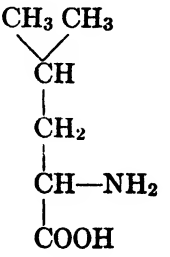
5. *d*-Valine, $C_5H_{11}NO_2$, or α-aminoisovaleric acid. (1901 Fischer.)



6. Norleucine, $C_6H_{13}NO_2$, or α-aminocaproic acid. (1913 Abderhalden and Weil.)



7. *l*-Leucine, $C_6H_{13}NO_2$, or α-aminoisocaproic acid. (1820 Braconnot.)



8. *d*-Isoleucine, $C_6H_{13}NO_2$, or α-amino-β-methyl-β-ethylpropionic acid. (1903, F. Ehrlich.)

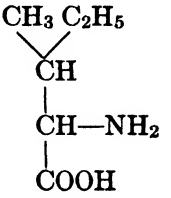


FIG. 3.

FIG. 4.

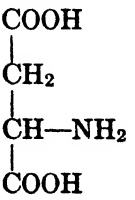
FIG. 3.—Tyrosine. Magnification about 54 times. Crystal habit—thin needles and rods, aggregate into tufts and sheaves.

FIG. 4.—Cystine. Magnification about 45 times. Crystal habit—colorless hexagonal plates and prisms.

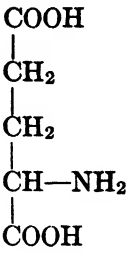
FIGURES 3 and 4 are reproductions from photomicrographs which were kindly furnished to the author by G. L. Keenan of the Bureau of Chemistry, United States Department of Agriculture. See *J. Biol. Chem.*, **62**, 163 (1924).

B. Monoaminodicarboxylic Acids.

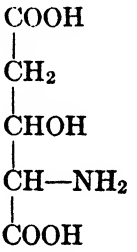
9. *l*-Aspartic acid, $C_4H_7NO_4$, or aminosuccinic acid. (1868, Ritthausen.)



10. *d*-Glutamic acid, $C_5H_9NO_4$, or α-aminoglutaric acid. (1866, Ritthausen.)

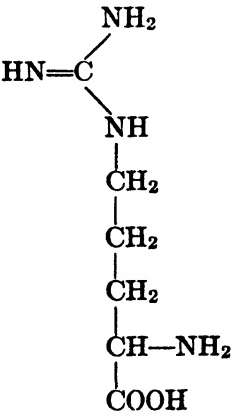


11. *d*-Hydroxyglutamic acid, $C_5H_9O_5N$, or α-amino-β-hydroxyglutaric acid. (1918, Dakin.)

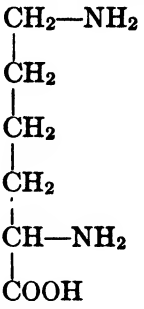


C. Diaminomonocarboxylic Acids.

12. *d*-Arginine, $C_6H_{14}N_4O_2$, or α-amino-δ-guanidine valeric acid. (1895, Hedin.)

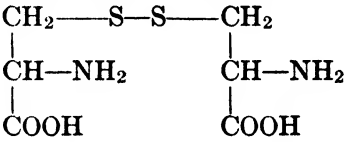


13. *d*-Lysine, $C_6H_{14}N_2O_2$, or α-ϵ-diaminocaproic acid. (1889, Drechsel.)

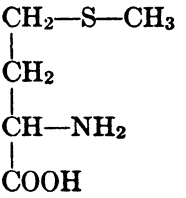


D. Sulfur-containing Amino Acids.

14. *l*-Cystine, $C_6H_{12}N_2O_4S_2$, or dicysteine, or di-(β-thio-α-aminopropionic acid). (1899, Mörner.[8])

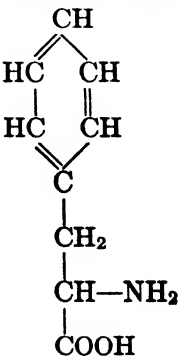


15. *l*-Methionine, $C_5H_{11}SNO_2$, or α-amino-γ-methylthiol-*n*-butyric acid. (1922, Mueller.)



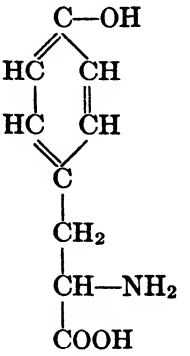
II. Aromatic Amino Acids.

16. *l*-Phenylalanine, $C_9H_{11}NO_2$, or α-amino-β-phenlypropionic acid. (1881, Schulze and Barbieri.)



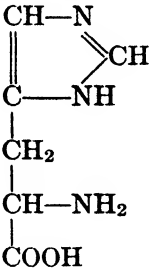
[8] First discovered by Wollaston in 1810.

17. *l*-Tyrosine, $C_9H_{11}NO_3$, or β-parahydroxyphenyl-α-aminopropionic acid. (1846, Liebig; 1849, Bopp.)

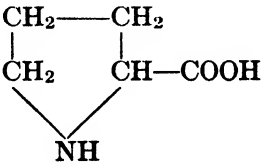


III. Heterocyclic Amino Acids.

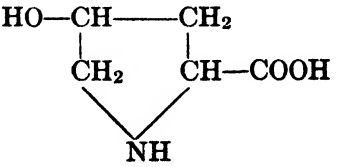
18. *l*-Histidine, $C_6H_9N_3O_2$, or β-imidazole-α-aminopropionic acid. (1896, Kossel, Hedin.)



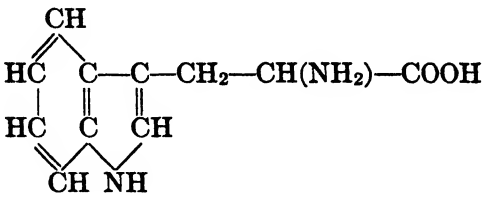
19. *l*-Proline, $C_5H_9NO_2$, or α-pyrrolidinecarboxylic acid. (1901, Fischer.)



20. *l*-Hydroxyproline (oxyproline), $C_5H_9NO_3$, or γ-hydroxy-α-pyrrolidinecarboxylic acid. (1902, Fischer.)



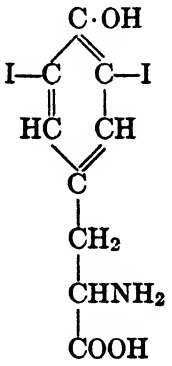
21. *l*-Tryptophane, $C_{11}H_{12}N_2O_2$, or β-indole-α-aminopropionic acid. (1901, Hopkins and Cole.)



In the tabulation given by Vickery and Schmidt, two iodine-containing amino acids are included. *Iodogorgoic* acid (diiodotyrosine) was first isolated by Drechsel (1896) [9] from the products of alkaline hydrolysis of the horny skeleton of the coral *Gorgonia Cavolinii*. Wheeler and Mendel [10] (1909) found it in the common sponge. It was later isolated by Harrington and Randall [11] from the thyroid gland, and Foster [12] obtained it among the products of the alkaline hydrolysis of partially purified thyroglobulin.

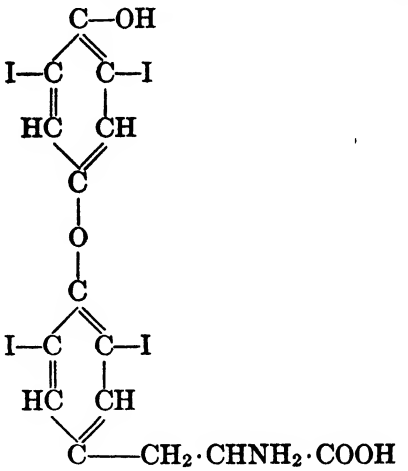
Thyroxine was discovered by Kendall [13] among the products of the alkaline hydrolysis of thyroid glands. Its chemical constitution was determined by Harington, and its synthesis was accomplished by Harington and Barger.[14]

22. Iodogorgoic acid, $C_9H_9NI_2O_3$, 3, 5-diiodotyrosine. (1896, Drechsel.)



[9] *Z. Biol.*, **33**, 96 (1896).
[10] *J. Biol. Chem.*, **7**, 1 (1909–10).
[11] *Biochem. J.*, **23**, 373 (1929).
[12] *J. Biol. Chem.*, **83**, 345 (1929).
[13] E. C. Kendall, Collected Papers, Mayo Clinic, **7**, 393 (1915).
[14] *Biochem. J.*, **20**, 293 (1926).

23. Thyroxine, $C_{15}H_{11}NI_4O_4$, β-[3, 5-diiodo-4-(3′, 5′-diiodo-4′-hydroxyphenoxy) phenyl]-alanine. (1915, Kendall.)



The foregoing list comprises the more important cleavage products of the protein molecule. The amino acids are present in varying proportions in different proteins, and though most proteins contain all the amino acids named (except those containing iodine) some are deficient in one or more of these. Gelatin is apparently made up of only fourteen or fifteen amino acids and contains little or no tyrosine and tryptophane. Glycine, tryptophane, and lysine are deficient or lacking in zein of corn. Then there are the protamins, which are made up of even fewer amino acids. For example, salmine yields on hydrolysis only valine, serine, proline, and arginine; sturine yields arginine, lysine, and histidine. The animal body, at least in the higher forms, is unable to synthesize certain amino acids, such as leucine, tryptophane, lysine, methionine, and histidine. These are required, however, in the formation of tissue and hence are essential for proper nutrition. The rôle of amino acids in nutrition will be considered in greater detail in a later chapter.

In addition to the amino acids that have been listed, others have been reported as products of protein hydrolysis. Among these are: α-amino-*n*-butyric acid, γ-hydroxy-α-aminobutyric acid, nor-valine, hydroxyvaline, *l*-isoleucine, hydroxylysine, dihydroxyphenylalanine, thiol-histidine, dihydroxypyrrolealanine, diaminoglutaric acid, diaminoadipic acid, dihydroxyaminosuberic acid, dibromotyrosine, citrulline, ornithine, etc. It has also been claimed that cysteine ($CH_2SH—CHNH_2—COOH$) may exist as such in the protein molecule.

Separation of Amino Acids. The method introduced by Fischer [15] for the separation of amino acids consists in converting them into esters

[15] E. Fischer, "Untersuchungen über Aminosäuren, Polypeptide und Proteine" (1899–1906), Berlin, 1906.

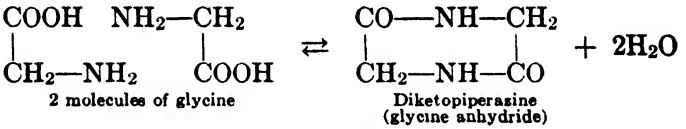
and subsequently separating the amino-acid esters by fractional distillation.

Amino acids may be separated electrolytically in a suitably constructed apparatus. The dicarboxylic acids, such as aspartic and glutamic, migrate to the anode; the basic acids, lysine, arginine, and histidine, concentrate at the cathode. Amino acids form salts with the heavy metals and may be separated as such. They also react with a large variety of other reagents to form insoluble crystalline derivatives, benzoates, picrates, picrolonates, etc.

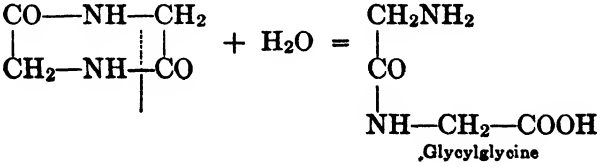
The products of acid hydrolysis of proteins may also be separated by extraction with various solvents, such as butyl and ethyl alcohols. This is the basis of a method first suggested by Dakin.[16]

In determining the proportions of mono- and diamino acids, the protein is hydrolyzed by boiling with hydrochloric acid. An insoluble residue, which may be removed by filtering, consists largely of so-called *humin* which contains nitrogen. The amide and ammonia nitrogen is determined by distillation with magnesium oxide *in vacuo* at 40° C. The diamino acids are precipitated with phosphotungstic acid, and the amount of nitrogen in the precipitate as well as in the filtrate is determined by Kjeldahl's method. The nitrogen content of the filtrate represents the monoamino acids.

The Synthesis of Polypeptides. Glycine, as the ethyl ester, in an aqueous solution forms an anhydride:

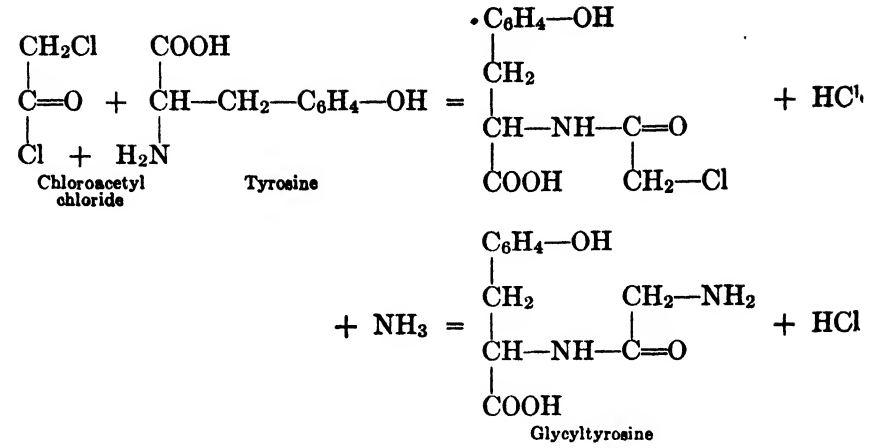


On boiling the anhydride with concentrated hydrochloric acid, Fischer obtained the dipeptide glycylglycine:

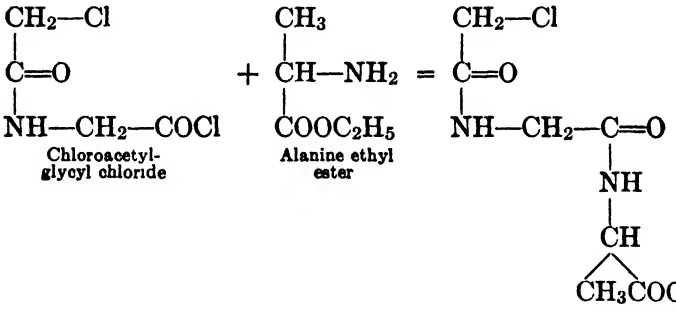


Another method devised by Fischer for the synthesis of a dipeptide consists in treating an amino acid with an α-halogen acyl radical. When the resulting compound is treated with ammonia a dipeptide is formed as represented by the following equations:

[16] *Biochem. J.*, **12**, 290 (1918); *J. Biol. Chem.*, **44**, 499 (1920).

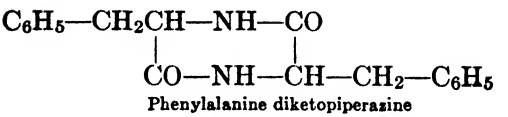


The acid chloride of the halogen acyl derivative of an amino acid reacts with amino-acid esters as follows:

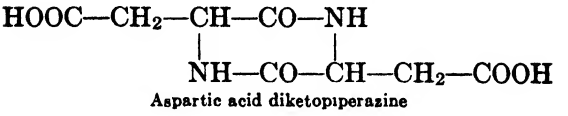


The ester group of the resulting compound may be hydrolyzed, and on subsequent treatment with ammonia, a tripeptide, diglycylalanine, is formed. By these and similar methods, Fischer and his followers have synthesized a variety of polypetides from amino acids. Fischer prepared a chain compound of as many as eighteen amino acids, namely, *l-leucyl-triglycyl-leucyl-triglycyl-leucyl-octoglycyl-glycine.* Later, Abderhalden synthesized a polypeptide chain composed of nineteen amino acids. The more complex synthetic polypeptides have many points of resemblance to the proteins. They are non-diffusible through a parchment membrane, give certain of the color reactions characteristic of proteins, and are precipitated from solution by tannic acid, phosphotungstic acid, and other protein precipitants.

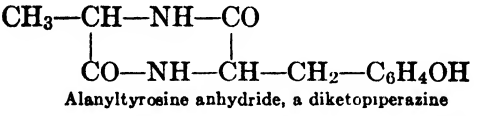
In the condensation of amino acids, compounds with cyclic structures have been formed. Glycine anhydride, or diketopiperazine, is a simple example. Substituted diketopiperazines may be obtained by the condensation of phenylalanine, aspartic acid, alanine and tyrosine, etc.



Phenylalanine diketopiperazine

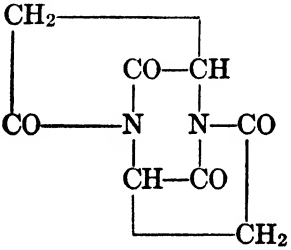


Aspartic acid diketopiperazine



Alanyltyrosine anhydride, a diketopiperazine

The dipeptide of aspartic acid (and glutamic acid) yields yet another type of cyclic compound (Blanchetier),[17] consisting of three condensed rings, as follows:



These, and a variety of other cyclic compounds, which have been obtained by the condensation of two or more amino acids, and which some workers claim to have isolated also among the products of protein hydrolysis, have acquired considerable interest in recent years in connection with various theories of protein structure that have been proposed.

Reactions of Amino Compounds. Nitrous acid reacts with amino compounds as represented by the equation:

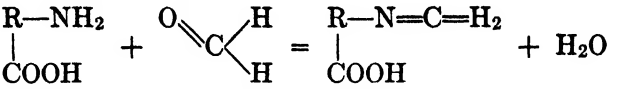
$$R{-}NH_2 + HNO_2 = ROH + H_2O + N_2$$

This reaction is the basis of the Van Slyke method [18] for determining the free amino groups in protein. Since hydrolysis of the protein molecule results in the development of a larger number of free amino (NH_2) groups, the method may be employed in following the progress of protein digestion.

The reaction between amino acids and formaldehyde is the basis of Sørensen's formol-titration method [19] which may be employed in deter-

[17] *Bull. Soc. chim. biol.*, **6**, 854 (1924).
[18] *J. Biol. Chem.*, **12**, 275 (1912).
[19] *Z. physiol. Chem.*, **64**, 120 (1909).

mining the number of free carboxyl groups. The reaction is adjusted to a definite alkalinity. Formaldehyde is then added to combine with the amino group, forming, according to Sørensen, a methylene derivative as represented by the equation:

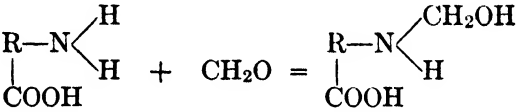


The effect of the basic amino group having been abolished, titration with standard alkali to the original reaction of the solution gives a measure of the number of free carboxyl groups.

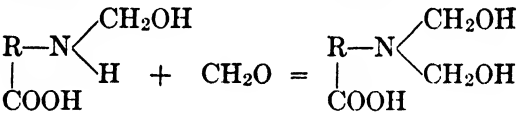
According to Harris,[20] the action of formaldehyde is to increase the acid ionization constant of the amino acids and is not due to reaction with the amino group.

The foregoing equation is considered inaccurate by Balson and Lawson [21] and Levy and Silberman[22], whose work apparently proves that an amino acid may combine with either one or two formaldehyde groups, as follows:

(1)

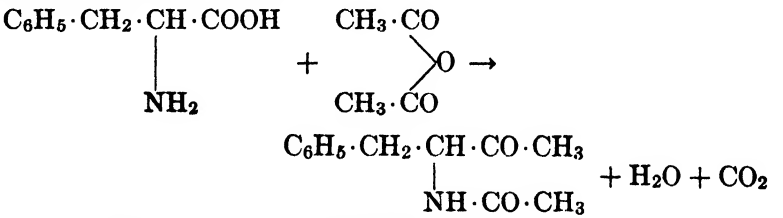


(2)



Imino groups react with only one molecule of formaldehyde.

A reaction which appears general to α-amino acids has been described by Dakin and West.[23] On warming amino acids with acetic anhydride and pyridine, carbon dioxide is evolved and two acetyl groups are introduced, one attached to nitrogen and one to carbon. The compounds have the general formula $R \cdot CH \cdot (NH \cdot COCH_3) \cdot COCH_3$, and are derivatives of acetylaminoacetone. The reaction with phenylalanine may be represented as follows:



[20] *Proc. Roy. Soc. (London), B,* **95**, 500 (1923-24).
[21] *Biochem. J.*, **30**, 1257 (1936).
[22] *J. Biol. Chem.*, **118**, 723 (1937).
[23] *J. Biol. Chem.*, **78**, 91, 745 (1928); see also P. A. Levene and R. E. Steiger, *ibid.*, **79**, 95 (1928).

The function of the pyridine appears to be catalytic. Proline and alkylamino acids do not react in this way, but undergo simple acetylation.

Reaction of Amino Acids with Acids and Bases; The "Zwitter Ion" Hypothesis. It has been customary to consider amino acids as amphoteric electrolytes, reacting as bases in the presence of acids and as acids in the presence of bases. In accord with the usual conception, such substances are supposed to be dissociated into ions either on the acid or basic side of a certain critical hydrogen-ion concentration, the "isoelectric point." As applied to amphoteric electrolytes, the isoelectric point has been defined as that point at which the ionization of the ampholyte is at a minimum. The following values represent the isoelectric points of certain of the amino acids (after Michaelis).[24]

Amino Acid	Isoelectric Point, *p*H
Arginine	10 52
Lysine	9 52
Leucine	6.05
Glycine	6 09
Alanine	6 21
Histidine	7 21
Phenylalanine	5 36
Tyrosine	5 41

The classical theory of isoelectric amino acids as amphoteric electrolytes may be exemplified in the case of glycine as in Formula I. Opposed to this concept is the more recently developed view known as the "zwitter-ion hypothesis." [25] The difference between the two theories is indicated by the following two formulas for isoelectric glycine.

I (Classical) II (Zwitter ion)

Isoelectric glycine

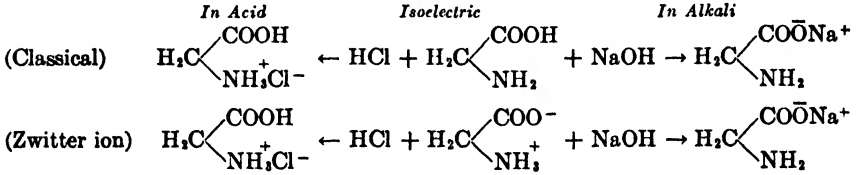
Formula I represents the molecule in a condition in which it is not dissociated either as an acid or as a base. The neutrality of the molecule is thus assumed to be due to the absence of dissociation, or at most to *minimal* and equal acid and basic dissociation. Formula II likewise represents a neutral molecule, but this neutrality is assumed to be due to the *complete* and simultaneous ionization of the acid and basic groups.

Although the zwitter-ion hypothesis represents a significant and

[24] "Die Wasserstoffionenkonzentration," Berlin, 1922, p. 60; compare with data given by E. J. Cohn in *Ergeb. Physiol.*, **33**, 834 (1931).

[25] For a fuller exposition of this hypothesis, the reader is referred to the following: N. Bjerrum, *Z. physik. Chem.*, **104**, 147 (1923); L. J. Harris, *Biochem. J.*, **24**, 1080 (1930); H. Borsook and D. A. MacFadyen, *J. Gen. Physiol.*, **13**, 509 (1930).

fundamental difference in mechanism, it is in agreement with the classical theory as regards the products which result from the addition of either acid or base. This is indicated by the following formulas:



The Synthesis of Protein in Nature. Our knowledge concerning the synthesis of protein in nature is very limited. Evidence has been adduced, however, to show that the animal cell is capable of synthesizing certain amino acids, such as glycine, alanine, and serine. It seems that in the lower organisms, such as the yeasts and bacteria, the synthesis of the aromatic and heterocyclic amino acids may be accomplished, and hence protein synthesis in these organisms occurs by the utilization of carbohydrates and simple sources of nitrogen.[26] In the higher organisms, however, the amino-acid supply is largely exogenous in origin. Animal life depends on the plants for its nitrogen supply. The dependence may be a direct one as in herbivorous animals, or it may be somewhat more remote as in the carnivorous animals.

For the most part, protein synthesis takes place in the leaves of plants from nitrogen supplied to the plant in the form of simple nitrogen-containing salts, the most important of which are the nitrates. A great many factors determine the growth of plants and their capacity to form proteins and other foodstuffs. It is obvious that the supply of nitrogen to the plant is an essential factor. Much of the nitrogen is absorbed by the plant in the form of nitrate and is reduced to nitrite under the influence of sunlight. The same effect may be achieved by exposure of potassium nitrate to the rays from a quartz mercury-vapor lamp. Further reduction of the nitrite doubtless occurs. Baudisch [27] has shown that the exposure of mixtures of potassium nitrite and methyl alcohol in aqueous solution to diffused daylight and ultraviolet light results in the reduction of the nitrite to hyponitrite and the oxidation of the methyl alcohol to formaldehyde. The two products thus formed react to give the potassium salt of formyhydroxamic acid:

$$KNO_2 + CH_3OH = KNO + HCHO + H_2O$$

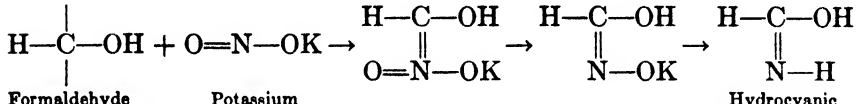
$$KNO + HCHO = H{-}C(=N{-}OK){-}OH$$

[26] See for example: E. Abderhalden and P. Rona, *Z. physiol. Chem.*, **46**, 179 (1905); S. Tamura, *ibid.*, **88**, 190 (1913); C. E. Skinner and C. G. Gardner, *J. Bact.*, **19**, 161 (1930); Skinner, *ibid.*, **28**, 95 (1934).

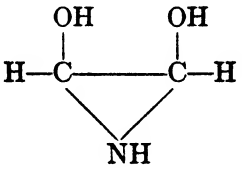
[27] *Ber.*, **44**, 1009 (1911); **49**, 1176 (1916); **51**, 793 (1918); see also O. Baudisch and L. A. Welo, *Chem. Rev.*, **15**, 1 (1934).

Another important factor which determines the synthesis of proteins is the available supply of carbohydrate. The synthesis of proteins in the plant can take place in the dark, provided there is an adequate supply of carbohydrate and potassium. It can be seen therefore that radiant energy may have only an indirect effect on protein formation, for it will be recalled that the formation of carbohydrates is the result of photosynthetic reactions.

According to Treub,[28] hydrocyanic acid is the first recognizable product of nitrogen assimilation in the plant. More recently, Baly, Heilbron, and Hudson [29] have reported that formaldehyde may react with potassium nitrate or nitrite to yield potassium formhydroxamate, a compound which on hydrolysis and subsequent reduction yields a hydrate of hydrocyanic acid. These changes may be represented as follows:



Formhydroxamic acid is said to condense with activated formaldehyde to yield an unstable ring compound:



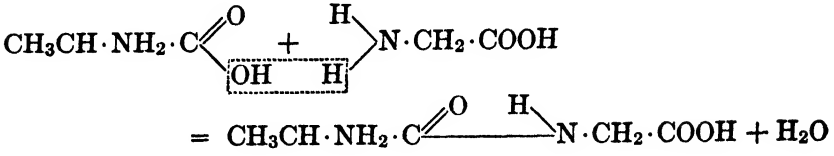
which by molecular rearrangement may conceivably yield glycine.

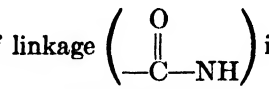
On the other hand, Björkstén [30] in a remarkable study of protein synthesis in nitrogen-starved wheat seedlings found that hydrocyanic acid was not used as a source of nitrogen. Nitrites and ammonium salts of aliphatic organic compounds were, however, well utilized.

The Structure of Proteins and the Types of Linkage in the Protein Molecule. The pioneer studies of protein structure by Hofmeister [31] and Fischer [32] led to the formulation of the hypothesis that amino acids are linked together through the amino group of one amino acid and the carboxyl group of another, forming long chains of amino acids. The

[28] *Ann. Jardin Botan. Buitenzorg*, **13**, 1 (1896).
[29] *J. Chem. Soc.*, **121**, 1078 (1922).
[30] *Biochem. Z.*, **225**, 1 (1930).
[31] *Ergeb. Physiol.*, **1**, 759 (1902).
[32] *Ber.*, **35**, 1095 (1902).

union between the two constituent amino acids in the dipeptide, alanylglycine, illustrates this form of combination.



This type of linkage  is called the *peptide bond.* The view that it is the principal linkage existing between amino acids in the protein molecule is based on the following considerations, as enumerated by Vickery and Osborne: [33]

1. Native protein itself contains very little amino nitrogen, but the end-products of protein hydrolysis contain larger amounts. The peptide bond type of union readily accounts for this.

2. The biuret reaction (p. 116) is given by many substances which contain this group, and this reaction is characteristic of proteins and their decomposition products, the proteoses. It disappears on complete hydrolysis. This strongly suggests the presence of the peptide bond in proteins and their partial hydrolysis products.

3. A number of condensation products of amino acids have been prepared which contain this group. Many of these give the biuret reaction.

4. The peptide union is also encountered in other naturally occurring substances, for example, hippuric acid.

5. The synthetic polypeptides obtained by Fischer from the natural isomers of optically active amino acids are hydrolyzed by the enzymes of the digestive tract.

6. Polypeptides have frequently been found among the products of incomplete hydrolysis of proteins.

7. During the hydrolysis of proteins, whether by acids or enzymes, amino groups and carboxyl groups are progressively liberated at an approximately equal rate.

8. Hydrolysis of proteins occurs without material change in the hydrogen-ion concentration of the solution. This is consistent with the view that equivalent amounts of amino and carboxyl groups are thereby produced.

9. Pepsin alone liberates as a rule about 20 per cent of the total amount of amino nitrogen which can be obtained by the complete hydrolysis of a protein. Erepsin acting on a peptic digest can liberate as much as 70 per cent more. Since there is every reason to believe that erepsin acts only upon peptide bonds, it is obvious that by far the greater part

[33] *Physiol. Rev.*, 8, 393 (1928).

of the total possible amino nitrogen of a protein has its origin in such bonds.

However, certain facts seem to point to the possibility that the protein molecule is not merely a single large polypeptide. This type of structure is believed to be inconsistent with the changes which protein undergoes in the process of denaturation by alcohol or heat. Nor is it possible to explain the insolubility in water of many proteins on the basis of a polypeptide structure. An even greater obstacle in accepting the peptide bond as being the sole link between amino acids is the behavior of pepsin toward polypeptides. Pepsin does not act on polypeptides, nor for that matter on any synthetic products formed from amino acids. In fact, it is not known which bonds in the protein molecule are attacked by this enzyme.

To explain these and other peculiarities in the behavior of proteins, a number of theories regarding their structure have been advanced. These theories, it should be made clear, do not challenge the validity of the Hofmeister-Fischer hypothesis, in its fundamental aspect, namely, the significance of the peptide bond; they pertain more to other general features of protein structure, such as the length of the polypeptide chains, the arrangement of amino-acid residues, and the relative importance of secondary valence forces. Other theories, based partly on X-ray data, are concerned more with the geometrical relations within the molecule.[34]

Among the many theories may be mentioned Abderhalden's *diketopiperazine hypothesis*,[35] according to which the protein molecule is built up of a number of diketopiperazine complexes (p. 95) which are associated or held together by forces of secondary or latent valence.[36]

Sørensen[37] holds that at least certain proteins are in a molecular condition such as may be represented by the formula $A_xB_yC_z \ldots$, in which A, B, C, and so on, represent components of a definite character

[34] Reference has been made to the review by H. B. Vickery and T. B. Osborne[33] of the hypotheses of the structure of proteins, *Physiol. Rev.*, **8**, 393 (1928). Another review by Vickery, "Recent Contributions to the Theory of Protein Structure," *Yale J. Biol. Med.*, **4**, 595 (1932), is recommended to the student, as well as the review by E. Klarmann, *Chem. Rev.*, **4**, 51 (1927), and the more recent contributions by R. J. Block, "On the Nature and Origin of Proteins," *Yale J. Biol. Med.*, **7**, 235 (1935), and "Proteins of the Nervous System, Considered in the Light of the Prevailing Hypotheses on Protein Structure," *ibid.*, **9**, 445 (1937). These reviews contain references to the work of Fischer, Kossel, Hofmeister, Abderhalden, Fodor, Bergmann, Karrer, and others who have attempted to solve the baffling problem of the constitution of the protein molecule.

[35] *Naturwissenschaften*, **12**, 716 (1924); *Z. physiol. Chem.*, **128**, 119 (1923); *ibid.*, **139**, 169, 181 (1924).

[36] According to Werner's conception there are two kinds of valence, one which he termed "primary" valence, and the residual attraction left over after the primary valence is saturated, which Werner called "secondary" valence. This idea of auxiliary or potential valence forces may be harmonized, with certain modifications, with the more modern views of atomic structure.

[37] *Compt. rend. trav. lab. Carlsberg*, **18**, No. 5 (1930); see also Editorial, *J. Am. Med. Assoc.*, **99**, 908 (1932).

and composition (e.g., polypeptides), and x, y, z, and so on, indicate the number of such components in the complexes. Within each component, strong chemical bonds unite the atoms or groups of atoms, whereas the various components are linked to each other to form the larger complex by relatively weak, residual valences. This concept of proteins as reversibly dissociable component systems has received support from the work of Block [38] with serum protein (p. 234), as well as from Svedberg's molecular-weight studies. Svedberg [39] believes that in *solution*, a protein, as for example egg albumin, does not possess the molecular weight which characterizes the crystalline product. The implication is that, during the process of purification of a protein, it is built up from particles of a relatively small size. By means of the ultracentrifuge Carpenter [40] has more recently shown that casein (molecular weight 96,000) in dilute solution may dissociate into molecules one-third the original size. The process could be reversed by increasing the concentration of the casein. It would seem, therefore, that at least certain proteins may have a variable molecular size and be capable of molecular dissociation as well as of aggregation. Globulin of wheat flour has also been shown to polymerize in concentrated solution and to dissociate on dilution (Krejci and Svedberg [41]).

The numbers of molecules of amino acids seem to be related in a simple arithmetical ratio, as determined from computations based on analytical data (Bergmann and associates [42]). In gelatin, for example, glycine represents one-third of all the amino acids, while proline represents one-sixth, and hydroxyproline one-ninth. The frequency of occurrence of amino acids has also been determined for egg albumin, hemoglobin, fibrin, silk fibroin, and other proteins. Bergmann has asserted that this relationship is not fortuitous, but must represent a regularity in the structure of the protein itself. He and Niemann have indeed proposed the hypothesis that "in every protein each amino acid residue is distributed throughout the entire peptide chain at constant intervals, i.e., each amino acid residue recurs with a characteristic whole number frequency."

X-ray methods used in the study of the internal structure of crystals and molecules have been applied recently to the problem of protein constitution, and the results seem to support, at least in part, the amino-acid chain system hypothesis. There is also evidence that protein readily undergoes intramolecular rearrangement. When hair, a rich source of keratin, is stretched, the X-ray photograph obtained is different from the normal one. This has been interpreted to mean that there are

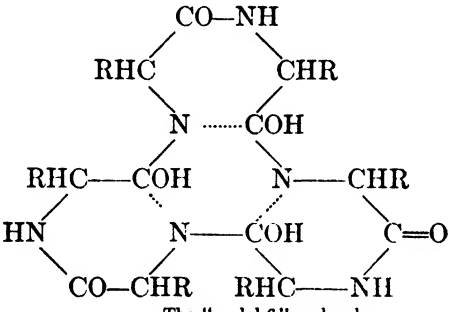
[38] *J. Biol. Chem.*, **103**, 261 (1933); *Yale J. Biol. Med.*, **7**, 235 (1935), **9**, 445 (1937).
[39] *Nature*, **128**, 999 (1931); *Chem. Rev.*, **20**, 81 (1937).
[40] *J. Am. Chem. Soc.*, **57**, 129 (1935).
[41] *Ibid.*, **57**, 1365 (1935).
[42] Bergmann, "Proteins and Proteolytic Enzymes," *Harvey Lectures*, **31**, 37 (1935-36); Bergmann and Niemann, *J. Biol. Chem.*, **115**, 77 (1936), **118**, 301 (1937).

Supercontracted Keratin
β-Keratin
10.2 Å
or
α-Keratin
5.1 Å
or

two forms of keratin, α-keratin and the denatured longer modification, β-keratin (Astbury and Woods [43]). A third, or supercontracted, form of keratin is obtained when hair that is moist is held under tension and heated, and then the tension relaxed before setting has taken place. These intramolecular transformations are reversible processes. The three forms of keratin are represented by formulas on page 103.

These polypeptide chains are supposed to lie along the fiber axis, either parallel to it, or spirally inclined at some constant angle. The chains are believed to be bound to each other by covalent, or possibly primary valence linkages, forming bundles, or micelles.

Wrinch [44] has proposed the view that proteins are composed of closed polypeptide chains, consisting of 2, 6, 18, 42, 66, 90, 114, 138, 162, . . . $(18 + 24n)$ amino acid residues. It is pointed out by Wrinch that, since all amino acids known to occur in protein are α- derivatives, they may be folded in hexagonal arrays. The general arrangement of the amino acids may be illustrated by the following formula of a cyclol of six amino-acid residues: [45]



The "cyclol 6" molecule

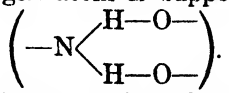
Information concerning types of linkage in protein, other than the peptide bond, is rather meager and indefinite. Within the amino acids themselves, there is the guanidine bond ($NH—CH_2$) of arginine and the disulfide linkage (S–S) of cystine. It is not improbable that the latter may form a side chain link between different polypeptide chains through cysteine residues, assuming that such exist. Between the free amino group of a diamino acid, such as lysine, and the carboxyl group of a dicarboxylic acid, such as aspartic, a salt linkage

$$(\ldots CH_2 \cdot NH_3^+ \ {}^-OOC \cdot CH_2 \ldots)$$

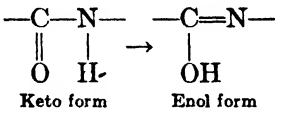
[43] W. T. Astbury and H. J. Woods, *Nature*, **126**, 913 (1930); **127**, 663 (1931); *Trans. Roy. Soc. (London), A*, **232**, 333 (1933); Astbury, *Nature*, **137**, 803 (1936).

[44] *Nature*, **137**, 411 (1936); **138**, 241, 651, 758 (1936).

[45] From the formula it is seen that Wrinch assumes a lactam-lactim rearrangement. Lack of space prevents a consideration of the basis and details of Wrinch's "cyclol" theory. The student is therefore referred to the original papers.[44]

may form. As a refinement of this side-chain, salt-like linkage, the existence of the "hydrogen bond" has been postulated by Mirsky and Pauling.[46] In this, each nitrogen atom is supposedly attached by a hydrogen to two oxygen atoms  . There is a little or no evidence for the presence of ether and imide bonds between amino acids in the protein molecule.

It is probable that the peptide group is capable of rearrangement from the keto to the enol form, as follows:



Molecular Weights of Proteins. The physical and chemical properties of the proteins have stimulated much interest in the question of their molecular weights. Various methods have been employed in an attempt to determine the relative size of the molecules of various proteins. The minimal weight of a protein molecule may be calculated from analytical data by assuming the presence, in the molecule of protein, of but one molecule of a given amino acid. The molecular weight of hemoglobin has been calculated thus from the amino-acid content, and also from data obtained of its iron content and of the oxygen content in oxyhemoglobin. It has also been shown that 16,721 grams of hemoglobin combine with one mole of carbon monoxide. Consequently this value was formerly taken for the minimal molecular weight of hemoglobin.

Determination of the equivalent combining weights of various proteins with acids and bases may yield useful information, especially when compared with calculations of their molecular weights based on the analyses of such elementary constituents as iron (in the case of hemoglobin), copper (in hemocyanin), sulfur, and phosphorus.

Direct determination of the molecular weights of proteins by osmotic-pressure methods has yielded results approximating those obtained by analytical methods. For the determination of the relative size of protein molecules, methods of ultrafiltration, electrophoresis, and diffusion have been found very useful. Svedberg and followers[47] have determined the molecular weights of proteins by measuring their sedimentation velocities in an ultracentrifuge. The following are illustrative data:

[46] *Proc. Natl. Acad. Sci. U. S.*, **22**, 439 (1936).

[47] T. Svedberg and associates: series of papers in *J. Am. Chem. Soc.*, **46**, (1924), to date; T. Svedberg, "Protein Molecules," *Chem. Rev.*, **20**, 81 (1937). The student is also referred to the review of the subject by A. Roche, *Bull. soc. chim. biol.*, **17**, 704 (1935). This includes a good account of the various physical and chemical methods used in the determination of the molecular weight of proteins.

Protein	Estimated Molecular Weight
Bence-Jones protein β	37,600
Egg albumin	43,800
Serum albumin (horse)	67,100
CO-hemoglobin (man)	63,000
Serum globulin (horse)	150,000
Edestin	303,000
Hemocyanin (*Palinarus*)	446,000
Thyroglobulin	696,000
Hemocyanin (*Helix pomatia*, main compound)	6,630,000

From these and other data it appears that the molecular weights of proteins are for the most part simple multiples of 35,100. Proteins of one group, including egg albumin and Bence-Jones protein, have molecular weights approximating this value. Those of another group, including hemoglobin and serum albumin, have molecular weights close to $2 \times 35{,}100 = 70{,}200$. Other proteins, for example, serum globulin, have molecular weights in the neighborhood of $4 \times 35{,}100 = 140{,}400$. Edestin, amandin, excelsin, etc., have molecular weights of about 281,000 ($8 \times 35{,}100$). In the case of certain proteins, there seem to be different molecular species, representing different grades of association and dissociation. Thus, two of the dissociation compounds of *Helix pomatia* hemocyanin have molecular weights of 502,000 and 719,000, while the main compound has a molecular weight of 6,630,000, or approximately $192 \times 35{,}100$. Lactalbumin, cytochrome C, and erythrocruorin are proteins of molecular weight approximating 17,600 ($\frac{1}{2} \times 35{,}100$).

One of the major scientific achievements of recent years has been the discovery by Stanley [48] that the virus of tobacco mosaic disease is probably a protein. This protein, purified by repeated crystallization, was found to be highly infectious, producing the disease in healthy tobacco and in other plants, such as the tomato. From the diseased tomato plant a protein was isolated in turn, which in its chemical and physical properties was indistinguishable from the protein virus of tobacco mosaic disease. This protein is believed to have the extraordinary molecular weight of about 17,000,000.

Behavior of Proteins as Electrolytes. In 1900 Hardy [49] demonstrated that particles of coagulated egg albumin were differently influenced by an electric current, depending on whether the reaction of the solution was acid or alkaline. In a slightly alkaline solution the protein moved from the cathode to the anode, whereas in the presence of acid the protein acquired a positive charge and migrated in the direction of

[48] W. M. Stanley, *Science*, **81**, 644 (1935); R. W. G. Wyckoff, J. Bisçoe, and Stanley, *J. Biol. Chem.*, **117**, 57 (1937); Stanley, *ibid.*, p. 325; H. S. Loring and Stanley, *ibid.*, p. 733; Stanley, *ibid.*, p. 755; I. B. Ericksson-Quensel and T. Svedberg, *J. Am. Chem. Soc.*, **58**, 1863 (1936).

[49] *Proc. Roy. Soc.*, **66**, 110 (1900).

the cathode. According to Hardy, the H^+ or OH^- ions become entangled within the colloid particle of protein, which thereby acquires a positive charge if there is an excess of H^+ ions (acid solution) or a negative charge if the OH^- ions are in excess (alkaline solution).

The explanation offered by Loeb[50] in 1904 was that proteins behaved like amphoteric electrolytes, reacting as bases in the presence of acids and as acids in the presence of bases. Owing to the presence of the amino group in the amino-acid molecule, this reacts with acids as though it were a basic substance. When placed in an alkaline solution, amino acids behave as though they were acids, because of the carboxyl group. Since the protein molecule likewise has at least one free amino group and one free carboxyl group, it will yield a protein cation in the presence of acids and will form protein chlorides, sulfates, etc. In the presence of bases it will yield a protein anion to form such compounds as sodium, potassium, or calcium proteinates. The protein molecule is obviously capable of electrolytic dissociation. The degree of dissociation of a protein and its capacity to combine with anions or cations is conditioned by the hydrogen-ion concentration, and for each protein the degree of dissociation is negligible at its *isoelectric point.*

The following are the isoelectric points of several familiar proteins (after Michaelis):[51]

Serum albumin	4.7
Serum globulin	5.4
Egg albumin	4.8
Edestin	6.9
Casein	4.7
Gelatin	4.7

Chemical Reactions of Proteins with Anions and Cations. Assuming that proteins are amphoteric electrolytes, they should combine with anions only on the acid side of the isoelectric point, and with cations only on the alkaline side. At its isoelectric point, the protein should combine with but negligible amounts of either acid or base. The correctness of this assumption was proved by Jacques Loeb[52] in a very ingenious manner:

Equal amounts of commercial powdered gelatin were brought to a different *p*H by treatment with varying concentrations of nitric acid. Silver nitrate was then added and the excess removed by washing with cold water. The gelatin was in turn dissolved and the solutions exposed in test-tubes to light (the previous manipulations having been carried

[50] "Univ. Calif. Pub. Physiol.," **1**, 149 (1904).

[51] "Die Wasserstoffionenkonzentration," Berlin, 1922. Compare with data given by C. L. A. Schmidt, in Harrow and Sherwin's "Textbook of Biochemistry," p. 191. According to Schmidt, the isoelectric point of serum albumin is 5.5 and of serum globulin 4.4. See also G. S. Adair, "The Chemistry of the Proteins and Amino Acids," *Ann. Rev. Biochem.*, **6**, 163 (1937).

[52] Jacques Loeb, "Proteins and the Theory of Colloidal Behavior," McGraw-Hill Book Co., New York (1924), p. 34; see also *J. Gen. Physiol.*, **1**, 449 (1920).

out in a dark room). As a result, all the gelatin solutions with a *p*H > 4.7 became opaque and then brown or black, while the solutions of *p*H < 4.7, i.e., from *p*H 4.6 and below, remained transparent even when exposed to light for months or years (Fig. 5). Hence it may be concluded that the cation Ag is only in chemical combination with gelatin when the *p*H is > 4.7. At *p*H 4.7, or below, gelatin is not able to combine with Ag ionogenically.

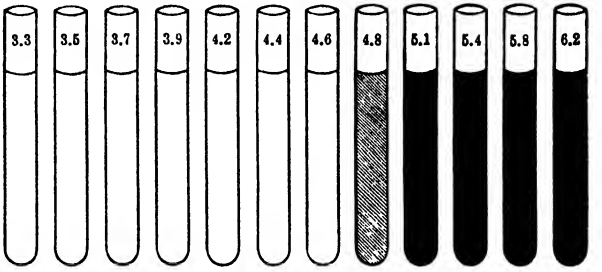


FIG. 5.—Proof that cations combine with proteins only on the alkaline side of the isoelectric point. Powdered gelatin brought to different *p*H was treated in a dark room with *M*/64 $AgNO_3$ and then washed with cold water to remove the silver not in combination with gelatin. The gelatin was liquefied, brought to a 1 per cent solution, and the *p*H was determined. The solutions were then poured into test-tubes and exposed to light In about half an hour the gelatin of *p*H > 4 7 was dark, while the gelatin of *p*H 4.7 or less remained permanently clear though exposed to light for over a year. The *p*H of each gelatin solution is marked at the head of each test-tube. (After J. Loeb, "Proteins and the Theory of Colloidal Behavior," McGraw-Hill, 1924 edition, p. 34.)

Loeb made similar tests with other cations, such as nickel and copper, and with basic dyes. Basic fuchsin and neutral red, after sufficient washing with cold water, stain only those gelatin solutions that have a *p*H above 4.7.

In order to bring out more fully the significance of the preceding observations, the gelatin molecule may be represented by the formula: [53]

$$\left[R\begin{matrix} NH_2 \\ COOH \end{matrix} \right]$$

in which the brackets indicate the inability of isoelectric gelatin to combine with either anions or cations. On the alkaline side of the isoelectric point, only the COOH reacts, in accordance with the following equation:

$$\left[R\begin{matrix} \overline{NH_2} \\ COOH \end{matrix} \right] + NaOH = \left[R\begin{matrix} \overline{NH_2} \\ COONa \end{matrix} \right] + H_2O$$

[53] This is based on Loeb's exposition. It may be revised in terms of the "zwitter-ion" concept without altering the fundamental significance or interpretation of the questions involved.

The sodium proteinate that is formed dissociates into a protein anion and a Na^+ cation:

$$\left[R\begin{matrix}\boxed{NH_2}\\ COONa\end{matrix}\right] \rightleftarrows \left[R\begin{matrix}\boxed{NH_2}\\ COO^-\end{matrix}\right] + Na^+$$

When other electrolytes are present, as $AgNO_3$, an interchange of cations takes place with the formation in this case of silver proteinate.

When protein on the acid side of the isoelectric point is treated with a salt it combines with the anion of the salt. Loeb demonstrated this by using potassium ferrocyanide and other salts. Gelatin was treated with $M/128$ $K_4Fe(CN)_6$ and subsequently washed. From the gelatin samples 1 per cent solutions were prepared and these allowed to stand for several days. It was found that the gelatin solutions with a pH < 4.7 turned blue (owing to the formation of ferric ferrocyanide) whereas the gelatin samples with a pH > 4.7 remained perfectly clear (Fig. 6). This is taken as evidence that the gelatin molecule enters

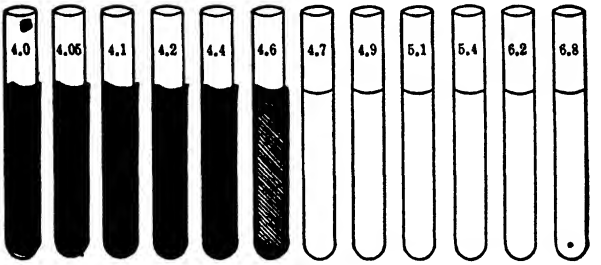


FIG. 6.—Proof that anions combine with proteins only on the acid side of the isoelectric point. Powdered gelatin solutions of different pH were treated with $M/128$ $K_4Fe(CN)_6$ and then washed with cold water. All the samples of gelatin solution of pH < 4.7 turned blue (through the formation of some ferric salt), while all the gelatin solutions of pH 4.7 or above remained colorless.

into chemical combination with the anion $Fe(CN)_6$ only when the pH is less than 4.7.

On the acid side of the isoelectric point, the amino group of the protein molecule behaves like ammonia in its ability to add an acid. This may be represented by the equation:

$$\left[R\begin{matrix}NH_2\\ \underline{COOH}\end{matrix}\right] + HCl = \left[R\begin{matrix}NH_2\cdot HCl\\ \underline{COOH}\end{matrix}\right]$$

The hydrochloride dissociates into a protein cation and an anion (Cl^-).

$$\left[R\begin{matrix}NH_3Cl\\ \underline{COOH}\end{matrix}\right] \rightleftarrows \left[R\begin{matrix}NH_3^+\\ \underline{COOH}\end{matrix}\right] + Cl^-$$

It is to be appreciated, of course, that the protein molecule very probably contains more than one reactive carboxyl and amino group.

Combination of Proteins with Acids and Bases. The conception that proteins undergo electrolytic dissociation is a radical departure from the views formerly held that because of the "colloidal" nature of proteins they did not react according to the "law of combining weights," that is, in *stoichiometric* proportions. The revision of this point of view was in no small measure the immediate outcome of Loeb's quantitative

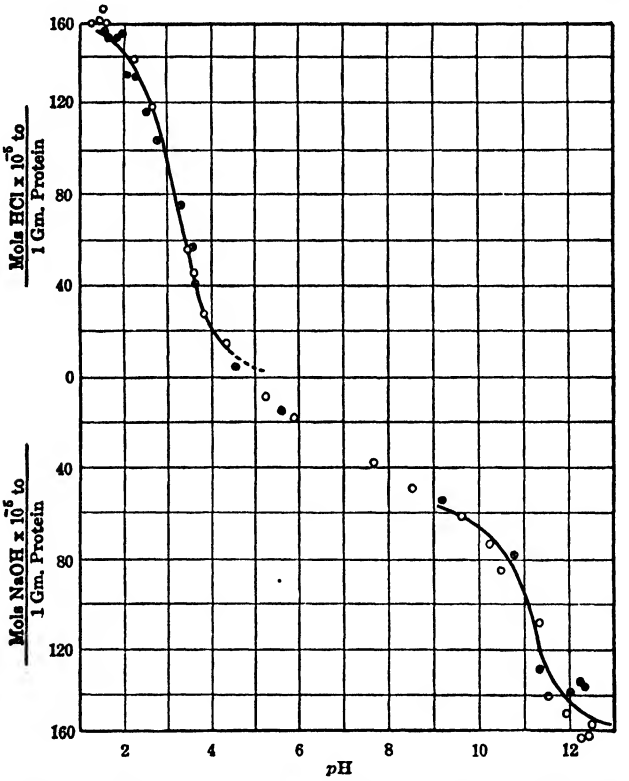


FIG. 7.—Titration curve of serum albumin, after E. J. Cohn.[64]

proof that proteins combine with acids and bases in definite and predictable proportions. Loeb pointed out that the earlier investigators failed to recognize the stoichiometric character of the reactions of proteins with acids and bases because methods for measuring the hydrogen-ion concentration of a protein solution had not then been devised and therefore its significance could not be appreciated.

On the acid side of its isoelectric point protein is combined with acid;

[64] E. J. Cohn, "The Physical Chemistry of the Proteins," *Physiol. Rev.*, **5**, 349 (1925); See also *J. Am. Chem. Soc.*, **59**, 509 (1937).

on the basic side with base. To produce a given change in the hydrogen-ion concentration (or *p*H) of a protein solution a definite quantity of either acid or base is required. In other words, it is possible to titrate protein with acids and bases and thereby determine the relation of the *p*H of the solution to the amounts of acid or base combined with the protein. If the results are plotted, a titration curve is obtained, the value of which is that it defines in an objective and accurate manner the acid- and base-combining properties of a given protein. This is shown for serum albumin by the titration curve in Fig. 7.

It is immaterial which of the strong mineral acids is used, whether HCl, HNO_3, or H_2SO_4. As was shown by Loeb's experiments, an identical number of cubic centimeters of 0.1 *N* acid was required in each case to produce the same change in the *p*H of a given protein solution (egg albumin, casein, gelatin, etc.). But, as was to be anticipated, in the case of 0.1 *N* H_3PO_4, which dissociates into H^+ and $H_2PO_4^-$ ions, three times as many cubic centimeters were required.

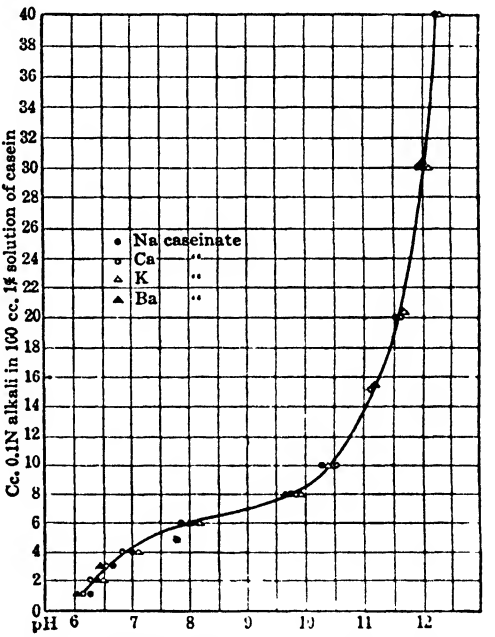


FIG. 8.—Ordinates are the cubic centimeters of 0.1 *N* NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$ in 100 cc. of 1 per cent solution of casein. Abscissas are the *p*H of the solution. The curves for the four alkalies are identical, proving that Ba and Ca combine with casein in equivalent proportion. (After Loeb, p. 69.)

The same principle holds when protein combines with bases (NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$). In the titration of protein (1 per cent casein, albumin, etc.) with 0.1 *N* solutions, the values (cubic centimeters of base required to bring the protein solution to a given *p*H) obtained for the different bases are found to lie on the same curve, as shown in Fig. 8.

Combination of Protein with Dyes. Rawlins and Schmidt[55] titrated casein, fibrin, gelatin, and edestin with certain basic dyes, methylene blue, saframine Y, and induline scarlet, and found that the union between protein and basic dye occurred in stoichiometric proportions.

[55] *J. Biol. Chem.*, **82**, 709 (1929).

Solubility of Proteins. Solutions in which the ultimate units are molecules or ions have been defined as true solutions, as distinguished from colloidal solutions in which the ultimate units are aggregates of molecules. Molecular aggregation or association is not limited, however, to colloidal particles. Many substances in the liquid state (water, acetic acid, methyl and ethyl alcohol) are not made up of discrete molecules, as can be demonstrated by comparing the molecular weights determined in the liquid state with those obtained in the vapor state. It has been generally assumed that proteins do not form true aqueous solutions but that the relatively large molecules form, by coalescence or aggregation, still larger particles, which remain in suspension.

The stability of colloidal particles suspended in water depends on the weak forces of repulsion due to the electrical double layer, of measurable potential difference, between each colloidal particle and the water. Diminution of the potential difference of the double layer below a certain critical value results in a coalescence of the particles into larger and larger aggregates, which settle out. The neutralization of the electrical charge of the colloidal particles may be accomplished even by relatively low concentrations of neutral salts, the effect being greater in the case of polyvalent ions having a charge opposite to that of the particles in suspension.

On the other hand, the stability of individual molecules and ions in true solution in water is due to the strong forces of attraction between the molecules or ions of the solute and of the solvent. Substances in true solution are not easily precipitated, therefore, by chemically non-reacting substances. Large concentrations of salts are required to precipitate substances from true solution and emulsoids. It is not a question of the neutralization of an electric charge, for the precipitation may be accomplished by an ion having a charge similar to that of the ion or molecule that is being removed from the solution.

We may now consider whether proteins in solution behave as crystalloids or as colloids. If, for precipitation, proteins required low concentrations of salts, this would be evidence for the suspensoid nature of their solutions. The fact that they require very large concentrations has been used in arguing for the emulsoid character of protein solutions, but this may also be used as an argument for the non-colloidal nature of protein solutions. In the precipitation of proteins, the active ion need not necessarily have a charge opposite to that of the protein.

Proteins are least soluble at the isoelectric point, a property which is also characteristic of other amphoteric electrolytes. It has been shown that true crystalloids, such as the amino acids, have minimum solubility at their isolectric points. In other words, amphoteric electrolytes and ionized protein salts are more soluble than the un-ionized molecules. Quantitative evidence that proteins may form true solutions has been furnished by Cohn and Hendry.[56] In this connection it may be recalled

[56] *J. Gen. Physiol.*, **5**, 521 (1922–23).

that at least certain proteins undergo molecular dissociation in dilute solutions, and aggregation, or polymerization, in concentrated solutions, the process being a reversible one (p. 102).

Denaturation and Coagulation. *Denaturation* is a process peculiar to proteins. The change from the fresh or "native" (undenatured) form to the denatured protein is not completely understood. Protein is denatured in the presence of water, by heating, exposure to ultraviolet light and to high pressures, shaking, addition of acid, alkali, alcohol, acetone, urea, and thiocyanate. In the neighborhood of the isoelectric point and within a fairly wide *p*H range protein will remain undenatured indefinitely. Brought outside of this range by either acid or alkali, denaturation occurs very rapidly. Whereas at ordinary temperatures (25° C.) the process of denaturation is slow, it may be greatly hastened by an increase in temperature, inasmuch as the velocity is accelerated 600 per cent for each 10° rise.

A striking change is in solubility. Native protein is soluble at its isoelectric point; denatured protein on the contrary is insoluble. It is definitely known that, even though the change is invisible, denaturation of protein in solution takes place at hydrogen-ion concentrations far removed from the isoelectric point, for when the solution is brought to the isoelectric point, denatured protein is precipitated. The precipitate may be redissolved by acid or alkali; hence the process of flocculation is *reversible*, and indeed it has been so considered for a long time. Denaturation, on the contrary, has been generally regarded as an *irreversible* process, a view that has been effectively challenged in recent years by the work of Anson and Mirsky.[57]

Of even more fundamental significance is the loss of certain specific properties, as has been stressed recently by Mirsky and Pauling.[58] Many native proteins have been crystallized and the crystal form found to be characteristic for each protein. All attempts to obtain denatured protein in crystalline form have failed. The specific immunological properties which distinguish most proteins are conspicuously diminished by denaturation. This loss in specificity is especially striking in the hemoglobins. The differences in crystal structure, absorption spectra, solubility, etc., which characterize hemoglobins from different animals, and even from closely related species, vanish with denaturation. As will be seen in a later chapter, certain enzymes (pepsin, trypsin) are known to be proteins and have been isolated in crystalline form. Denaturation causes the disappearance of their enzymatic activity.

These and other drastic effects of denaturation make the problem of the underlying structural changes one of great interest and importance. No clear view of the subject is possible from such isolated facts as the cleavage of sulfur from protein, or changes in the sulfhydryl groups, as

[57] *J. Phys. Chem.*, **35**, 185 (1931); *J. Gen. Physiol.*, **14**, 605, 725 (1931); **15**, 341 (1932).

[58] *Proc. Natl. Acad. Sci. U. S.*, **22**, 439 (1936).

occur in denaturation of protein by alkali, or the splitting off of ammonia when protein is denatured by heating. Among the many theories proposed is that denaturation, as well as coagulation, involves the condensation of opposite groups of adjacent molecules. This is borne out by the apparent reduction in the number of free amino and carboxyl groups, as determined by a comparison of the titration curves of native, denatured, and coagulated protein (Hendrix [59]).

Wu,[60] on the other hand, has offered the suggestion that in denaturation or coagulation the compact and orderly structure is disorganized. This is in line with the theory recently advanced by Mirsky and Pauling [58] that denaturation is associated with a very drastic change in the configuration of the protein molecule. The uniquely defined configuration which characterizes undenatured, or native, protein is lost. This disorganization of the molecule is believed to result from the cleavage of the side-chain hydrogen bonds, leaving the molecule free to assume any one of a large number of configurations.[61] Mirsky and Pauling stress the point that with the loss of the uniquely defined configuration there would be a loss of the specific properties of the native protein. For example, it would not be possible to grow crystals from molecules of varying shapes.

Chick and Martin [62] advanced the concept that the *coagulation* of a protein occurs in two distinct steps: the first, denaturation, is a chemical change; the second, precipitation or flocculation, depends on physical changes affecting the protein particles. Mirsky and Pauling explain coagulation as a process involving the joining together of adjacent protein molecules by means of side-chain hydrogen bonds.

[59] B. M. Hendrix and V. Wilson, *J. Biol. Chem.*, **79**, 389 (1928); M. Fay and Hendrix, *ibid.*, **93**, 667 (1931); compare with W. J. Loughlin, *Biochem. J.*, **27**, 99 (1933). For a general review of the subject, the reader is referred to W. C. M. Lewis, *Chem. Rev.*, **8**, 81 (1931).

[60] *Chinese J. Physiol.*, **5**, 321 (1931).

[61] Mirsky and Pauling's conception of the protein molecule is that it consists of one polypeptide chain which continues without interruption throughout. In certain cases the molecule may consist of two or more such chains. The chain is folded into a uniquely defined configuration, in which it is held by hydrogen bonds between peptide nitrogen and oxygen atoms and also between the free amino and carboxyl groups of the diamino and dicarboxyl amino acids.

The hydrogen bond, previously mentioned (p. 105), may be represented by N—H—O. It consists of a hydrogen atom which bonds two electronegative atoms together. The hydrogen bond is electrostatic in nature; it holds the bonded atoms more closely together than they would be if there were no bond. For further details concerning the nature of this bond, the reader is referred to W. M. Latimer and W. H. Rodebush, *J. Am. Chem. Soc.*, **42**, 1419 (1920); N. V. Sidgwick, "The Electronic Theory of Valency," Oxford University Press, 1929; J. D. Bernal and H. D. Megaw, *Proc. Roy. Soc. (London)*, *A*, **151**, 384 (1935). Mirsky and Pauling consider the side-chain bonds in proteins "to involve usually an amino and a carboxyl group, the nitrogen atom forming a hydrogen bond with each of two oxygen atoms and holding also one unshared hydrogen atom."

[62] *J. Physiol.*, **40**, 404 (1910); **43**, 1 (1911).

The reason why native proteins do not coagulate, as a rule, is that most of the side chains are unavailable, being in protected positions inside the molecule. As protein is denatured, more and more side chains become free and therefore the possibility of union is greatly increased, which means that coagulation occurs (more completely at the isoelectric point). However, coagulation may be unaccompanied by denaturation, as in myosin, which forms a coagulum on freezing, owing to the removal of water. Anson and Mirsky have demonstrated the complete reversal of coagulation of a protein (hemoglobin).

Regarding the significance of denaturation, physiologically, Anson and Mirsky have offered the suggestion that denaturation and its reversal are biological reactions which may be of importance in ordinary cellular processes.

The Colloidal Behavior of Proteins. It is beyond the scope of this book to enter into a detailed discussion of Loeb's work on the behavior of proteins. It may be asserted, however, that, as far as the proteins are concerned, there is no reason for distinguishing them either as colloids or crystalloids. With regard to their chemical reactions and solubility, the proteins behave like crystalloids. These constitute therefore the crystalloidal properties of proteins. On the other hand, the protein ion, on account of its large size, does not diffuse through membranes or gels which are permeable to smaller crystalloidal ions. This constitutes the colloidal property of the protein ion. Evidence has been adduced to show that the behavior of proteins may be explained on the basis of Donnan's theory of membrane equilibria, which applies to the equilibria established between ions on the two sides of a membrane impermeable to one of the ions. The effect of electrolytes on the swelling of proteins, osmotic pressure, membrane potentials, and the viscosity of protein solutions may be accounted for, according to Loeb and his followers, by application of Donnan's postulates.[63]

Hoffman and Gortner,[64] in an exhaustive study of the physicochemical behavior of the prolamins, concluded that a chemical type of combination between proteins and acids or bases occurs only between hydrogen-ion concentrations corresponding to *p*H 2.5 to 10.5. Working with a large variety of proteins belonging to this group, these authors found that the amount of acid or alkali bound at any hydrogen-ion concentration is dependent on the chemical composition of the protein. This may be taken as evidence of a chemical type of combination within this range of *p*H values. However, at hydrogen-ion concentrations higher than that represented by *p*H 2.5 or lower than that represented by *p*H 10.5, all the proteins, regardless of their chemical composition, combine with the same amount of acid or alkali, as the case may be.

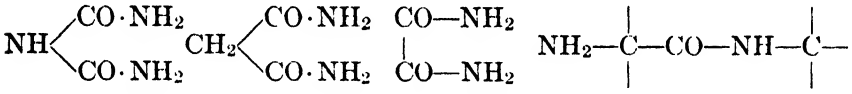
[63] See David I. Hitchcock's "Review of Proteins and the Donnan Equilibrium," *Physiol. Rev.*, **4**, 505 (1924).

[64] W. F. Hoffman and R. A. Gortner, "Colloid Symposium Monograph," Vol. 2, p. 209, etc., Chemical Catalog Co., New York. 1925.

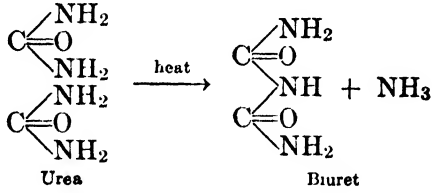
Moreover, with increases in hydrogen-ion concentration, protein salts, such as the chloride, increase in ionization, so that at a *p*H of 2.5 the protein chloride is highly ionized. But when the hydrogen-ion concentration is increased above *p*H 2.5, there is no further increase in the ionization of the protein salt. These and similar observations, according to Hoffman and Gortner, argue for the adsorption type of combination between proteins and bases or acids outside the *p*H range of 2.5 to 10.5.

Color Reactions of the Proteins. Most proteins exhibit characteristic color reactions when treated with certain reagents. The colors are due to specific linkages or to amino acids, and some reactions are specific for a particular amino acid. Hence these reactions may be employed in the qualitative characterization of proteins. Among the more familiar tests are the following:[65]

The Biuret Reaction. This is obtained by treating a protein solution first with strong alkali and then with a very dilute copper sulfate solution. A reddish violet to violet-blue color is produced. The reaction depends on the presence of the peptide linkage in the protein molecule. According to Schiff, any one of the following groups will give the test:



The reaction is so named because it is given by the substance biuret, which may be obtained by heating urea.



Urea Biuret

Millon's Reaction. The addition of Millon's reagent, a solution containing mercuric nitrate and nitrite in a mixture of nitrous and concentrated nitric acids, to protein solution, with heating, results in the formation of a brick-red precipitate. This reaction is due to the presence of the tyrosine group and is exhibited, as far as the red color is concerned, by substances, other than proteins, that contain the hydroxyphenyl group.

The Xanthoproteic Reaction. Nitric acid added to proteins produces a yellow color which deepens to an orange-yellow on the addition of alkali. The yellow color is due to the formation of nitrated benzene derivatives. The reaction depends on the presence of tyrosine, phenyl-

[65] Details of the procedure, which are here outlined briefly and incompletely, will be found in laboratory manuals of Physiological Chemistry.

alanine, and tryptophane. Some authors deny that phenylalanine gives the xanthoproteic reaction.

OTHER COLOR REACTIONS DUE TO TRYPTOPHANE

		Color
Acree-Rosenheim	Protein treated with dilute formaldehyde and layered with sulfuric acid.	Violet ring
Ehrlich's benzaldehyde test	Protein is boiled in concentrated hydrochloric acid. A few drops of *p*-dimethylaminobenzaldehyde dissolved in 10 per cent sulfuric acid are added.	Red to violet

COLOR REACTIONS OF PROTEINS SPECIFIC FOR OTHER AMINO ACIDS

	Amino acid		
Ehrlich's diazo test	Histidine, tyrosine	$NaNO_2$ solution mixed with sulfanilic acid dissolved in HCl. This solution is added to protein solution and the mixture is made ammoniacal.	Histidine, from orange to red color; tyrosine, light orange
Sullivan's test	Cystine Cysteine	In case of cystine, test depends on reduction to cysteine and reaction with 1,2-naphthoquinone-4-sodium sulfonate. Cysteine gives test more directly.	Red
Sakaguchi	Arginine (guanidine group)	NaOH added to protein solution and followed by α-naphthol and sodium hypochlorite.	Red

The biological value of proteins is largely determined by the proportions of their constituent amino acids. Deficiency in even one of the essential amino acids limits the value of a protein to the animal body. Osborne and Mendel and their followers in the United States as well as workers in other countries have studied this problem very exhaustively by means of feeding experiments performed on animals. This subject will be considered in another chapter. It is desirable at this point, however, to bring out the variations in the amino-acid composition of a number of proteins (p. 118).

Precipitation and Coagulation Reactions. The proteins are precipitated from solution by a large variety of substances. Among these are the neutral salts, such as sodium sulfate, magnesium sulfate, and ammonium sulfate. Large amounts of these are required for the separation of the proteins. The process is frequently spoken of as *salting out.*

The *salts of heavy metals,* such as those of copper, mercury, and lead, are good precipitants. Precipitation of proteins is also brought about by strong mineral acids. On the addition of nitric acid to a protein

TABLE XI

QUANTITATIVE COMPARISON OF AMINO ACIDS OBTAINED BY HYDROLYSIS FROM PROTEINS (in Per Cent)*

	Casein	Gelatin	Gliadin	Zein	Lactalbumin	Edestin	Salmine (Rhine salmon)	Silk-Fibroin (Italian)
Glycine	0.45	25 5	0.00	0 00	0.37	3.80	.....	36 0
Alanine	1 85	8.7	2 00	9.79	2 41	3.60	+	21 0
Valine	7 93	0 0	3 34	1 88	3 30	6 20	4.3	0.0
Leucine and Isoleucine	9 70	7 1	6 62	19 55	14 03	14 50	+	1 5
Proline	7 63	9 5	13.22	9 04	3.76	4.10	11.0	0 3
Hydroxyproline	0 23	14 1	?	?	?	?	.....	. ...
Phenylalanine	3 88	1 4	2 35	6 55	1 25	3 09	...	1 5
Glutamic acid	21 77	5 8	43.66	26 17	12 89	18 74	. . .	0 0
Hydroxyglutamic acid	10 50	0.0	2 4	?	10 00	?	.....	. ....
Aspartic acid	4 1	3.5	0 58	1 71	9 30	4 50	.....	.....
Serine	0 5	0 4	0 13	1 02	1.76	0 33	... .	. .
Tyrosine	4 5	0 01	1 61	3 55	1 95	2 13	. .	10 5
Cystine	?	?	0 45	?	1 73	1 00	. .	. .
Histidine	2 5	0 9	1 49	0 82	2 61	2 19	0 0	+
Arginine	3 81	8 2	2 91	1 55	3 47	14 17	87 4	1 0
Lysine	7.62	5 9	0 63	0 00	9 87	1 65	0 0	+
Tryptophane	1 50	0 00	1 0	0 00	2.40	1 50		
Methionine	3 1	0 97	...	2 5	.....	2 2	...	.. .
Ammonia	1.61	0 40	5 22	3 64	1 31	2 28	...	. .
Summation	93 18	92 38	87 61	87 77	82 20	85 98	110 5	73 4

* The data in this table are taken largely from a compilation by H B Vickery The analyses are combinations of what appear to be the best determinations by various chemists Cf. L B Mendel, "Nutrition—The Chemistry of Life," Yale Univ. Press, New Haven, 1923, p 115. See also Plimmer, "The Chemical Constitution of the Proteins," Part I, p. 111, etc.

solution, a ring of protein is formed at the junction of the acid and the solution (*Heller's test*).

The so-called *alkaloidal reagents* precipitate proteins more or less completely from slightly acid solution. Among these reagents are phosphotungstic acid, phosphomolybdic acid, tannic acid, picric acid, potassium mercuric iodide, and potassium bismuth iodide.

Ferrocyanic acid, trichloracetic acid, sulfosalicyclic acid, and dinitrosalicyclic acid are likewise efficient protein precipitants.

The Proteins as Foodstuffs. The proteins of vegetable origin play a very important part in animal nutrition. They have been very carefully and thoroughly studied by Osborne and his co-workers.[66]. The proteins found in various grains or cereals enter into the human dietary. Wheat contains gliadin, glutenin, and the albumin leucosin, in addi-

[66] T. B. Osborne, "The Vegetable Proteins," Longmans, Green & Co., 1924.

tion to a proteose. The mixture of gliadin and glutenin, when moistened, absorbs water to a greater degree than other proteins of the cereals. Rye contains a gliadin differing from that of wheat, and a glutelin. The swelling property of rye proteins is less than that of the proteins of wheat. Rye flour therefore yields a dough which is less elastic and less capable of becoming porous than wheat dough.[67]

The prolamin of barley is hordein. It differs markedly from the gliadin of wheat or rye. The remaining proteins of barley resemble those of wheat. The prolamin of corn is zein.

[67] See chapter by Carl L. Alsberg, "The Colloid Chemistry of the Cereals," in R. H. Bogue's "The Theory and Application of Colloidal Behavior," McGraw-Hill Book Co., New York, 1924.

CHAPTER V

SOURCES AND COMPOSITION OF FOODSTUFFS

It seems appropriate at this stage to consider briefly the sources of the protein, carbohydrate, and fat of our diet and also the composition of some of the more important articles of food.

The extensive statistical studies of Raymond Pearl [1] have provided us with very valuable information regarding the food consumption in the United States of America for the period 1911–1918. He has shown that, despite significant fluctuations in food production and food exports and imports, the total annual consumption of food shows remarkable uniformity from year to year. The following summary is based on data contained in his book, "The Nation's Food." [2]

Of the protein consumed in the United States, 47 per cent comes from primary food sources, i.e., food directly gathered or harvested, such as, for example, potatoes, fish, oysters; or food derived by process of manufacture from a raw plant product, such as, for example, wheat flour or cottonseed oil. The remaining 53 per cent is obtained from so-called secondary sources, i.e., edible products of animals obtained either directly (without involving the death of the producing animal), such as honey, eggs, milk, or derivatively (involving the death of the animal), such as meats.

Primary food sources provide only 18 per cent of the fats and secondary sources 82 per cent. Most of the fat of American diets is therefore derived from animal sources.

The condition is reversed in the case of carbohydrates, 95 per cent being furnished from primary and 5 per cent from secondary food sources.

Approximately 36 per cent of the total protein consumed comes from grain, 26 per cent from meat, and 20 per cent from dairy products. All but 18 per cent of the nation's food protein is therefore supplied by these three great commodity groups.

Meats furnish 51 per cent of the fat; dairy products, 27 per cent; vegetable oils and nuts about 12 per cent; and grains about 4 per cent.

Grains provide 56 per cent of the carbohydrate; sugars, 26 per cent, vegetables about 9 per cent; dairy products, 5 per cent; and fruits, 4 per cent.

Thirty-five per cent of the energy representing the total food consumption is derived from grains; 22 per cent from meats; 15 per cent

[1] R. Pearl, "The Nation's Food," Philadelphia, 1920; see also, R. Pearl, "Studies in Human Biology," Williams & Wilkins, Baltimore, 1924, Chap. XIV.

[2] It is conceivable that in the twenty-five years which have elapsed since the study by Pearl important changes may have taken place in the pattern of the "nation's food," but no nation-wide inclusive figures are available for the present conditions.

from dairy products; 13 per cent from sugars; about 5 per cent from vegetables; 5 per cent from vegetable oils and nuts; 2 per cent from poultry; and about 2 per cent from fruits.

Of the grains, wheat is by far the most important as a source of protein and carbohydrate, representing 29 per cent of the total protein and 42 per cent of the total carbohydrate consumption. Wheat provides the nation with 26 per cent of its food calories. However, it contributes only 1.8 per cent to the total fat consumption.

Corn ranks second among the grains, furnishing 5.55 per cent (6.4 per cent during the World War) of the protein, 1.9 per cent of the fat, 11 per cent of the carbohydrate and 7 per cent of the calories.

Rye, which is an important food in Russia and elsewhere in Europe, is little used in America. It supplies only 0.31 per cent of the protein, 0.03 per cent of the fat and about 0.8 per cent of the carbohydrate.

Dairy products furnish 20 per cent of the protein, 27.5 per cent of the fat, 5.5 per cent of the carbohydrate, and 15.26 per cent of the energy of the total food consumed.

Of the meats, beef is the most important from the standpoint of protein, representing 14.47 per cent of the total protein consumption. It contributes about 10 per cent to the fat consumption and provides 5.3 per cent of the total calories. Pork ranks first among all foods from the standpoint of fat; it provides 39.57 per cent of the total fat consumed in this country. It ranks fourth from the standpoint of protein, supplying 10.74 per cent of the total. The energy value represented by the pork consumption is 15.74 per cent, second only to wheat.

Of the vegetables, the potatoes are of greatest importance, representing 3.14 per cent of the total protein, 5.7 per cent of the total carbohydrate and 3.36 per cent of the total energy consumption.

As compared with other foods, fish occupy a relatively unimportant position in this country. Only 2.32 per cent of the total protein is derived from this source.

These figures represent gross consumption, being based on averages for the six years, 1911–12 to 1916–17, and do not take into account losses through wastage. Pearl has estimated the probable loss of edible food through wastage to be: for protein, about 5 per cent; fat, at least 25 per cent; carbohydrate, 20 per cent.

Composition of Some Foods.—With the relative importance of various foods in mind, we may now present the results of analyses of the protein, fat and carbohydrate contents of some of the more common foods.[3]

[3] The sources of the data contained in these tables are: Bulletin 28 (Atwater and Bryant), of the Office of Experiment Stations, U. S. Dept. Agr., Washington; H. C. Sherman, "Chemistry of Food and Nutrition," Macmillan, New York, 1928, Appendix B; U. S. Dept. Agr. Cir., 50 and 389. For further information the reader is referred to A. L. Winton and K. B. Winton, "The Structure and Composition of Foods," John Wiley & Sons, New York, Vol. 1, 1932, cereals, starch, oil seeds, nuts, oils, forage plants; Vol. 2, 1935, vegetables, legumes, fruits; Vol. 3, 1937, milk, eggs, meat, fish.

Unless stated otherwise, the data are based on the edible portion only. The inorganic constituents and vitamins will be considered elsewhere.

TABLE XII

COMPOSITION OF EDIBLE PORTION OF VARIOUS MEATS

	Per Cent Protein (N × 6.25)*	Per Cent Fat	Portion in Grams Equivalent to 100 Calories
Beef, chuck, lean	19.2	9	63
chuck, medium	18.6	16	45
chuck, fat	17 6	22	37
loin, lean	18 6	16	46
rib, medium	17 4	23	36
liver	20.4	4 5	78
sirloin steak	18 9	18 5	41
Porterhouse steak	21 9	20 4	37
Pork, chops	16 6	30 1	30
sausage	13 0	44 2	22
Bacon, smoked	10 5	64 8	16
Ham, fresh, lean	25 0	14 4	44
Lamb, chops, broiled	21 7	29 9	28
leg, roast	19 7	12 7	52
Mutton, leg	19 8	12 4	52
Veal, breast	20 3	11.0	56
cutlet	20 3	7 7	66

* The nitrogen content of most proteins is approximately 16 per cent, whence the factor 6.25.

TABLE XIII

COMPOSITION OF FISH AND OYSTERS

	Per Cent Protein (N × 6.25)	Per Cent Fat	Portion in Grams Equivalent to 100 Calories
Flounder	14.2	0 6	161
Haddock	17.2	0 3	140
Cod, salt	25.4	0 3	96
Halibut steaks	18.6	5.2	83
Mackerel	18.7	7.1	72
salt	21.1	22.6	35
Shad, whole	18.8	9 5	61
Salmon, whole	22.0	12.8	49
Whitefish	22.9	6.5	67
Oysters*	6.2	1.2	199

* Oysters contain about 3.7 per cent carbohydrate.

TABLE XIV

Composition of Poultry and Eggs

	Per Cent Protein (N × 6.25)	Per Cent Fat	Portion in Grams Equivalent to 100 Calories
Chicken, broilers	21.5	2.5	92
Fowls	19.3	16.3	45
Turkey	21 1	22 9	34
Eggs, uncooked	13.4	10.5	68

TABLE XV

Composition of Some Dairy Products

	Per Cent Protein (N × 6.25)	Per Cent Fat	Per Cent Carbohydrate	Portion in Grams Equivalent to 100 Calories
Milk, whole	3 3	4.0	5.0	145
condensed, sweetened	8 8	8 3	54 1	31
evaporated	6 7	8 1	10.3	71
Cream	2 5	18 5	4 5	50
Butter	1 0	85.0	....	13
Cheese, American pale	28 8	35 9	0.3	23
cottage	20.9	1 0	4 3	91
full cream	25 9	33 7	2 4	24
Swiss	27 6	34 9	1 3	23

TABLE XVI

COMPOSITION OF SOME FRUITS, BERRIES, AND NUTS

	Per Cent Protein (N × 6.25)	Per Cent Fat	Per Cent Carbohydrate	Portion in Grams Equivalent to 100 Calories
Apples	0.4	0 5	14 2	159
Bananas	1.3	0 6	22.0	101
Grapes	1 3	1 6	19 2	104
Grapefruit	0 6	0 1	12 2	193
Cherries, fresh	1.0	0.8	16 7	128
Figs, fresh	1 4	0 4	19.6	115
dried	4 3	0 3	74 2	32
Oranges	0.8	0 2	11 6	195
Olives, green	1 1	27 6	11.6	33
ripe	1 7	25 0	4 3	40
Peaches, fresh	0 7	0 1	9 4	242
Pears, fresh	0 6	0 5	14 1	158
Strawberries	1 0	0 6	7 4	269
Blackberries	1 2	1 1	7 8	160
Blueberries	0 6	0 6	13 9	146
Raspberries, red	1 0		12 6	184
Almonds	21 0	54 9	17 8	15
Brazil nuts	17 0	66 8	7 0	14
Peanuts	25 8	38 6	24 4	18
Walnuts (California or English)	18 4	64 4	13 0	14

TABLE XVII

COMPOSITION OF GRAIN PRODUCTS AND CEREALS

	Per Cent Protein (N × 6.25)	Per Cent Fat	Per Cent Carbohydrate	Portion in Grams Equivalent to 100 Calories
Flour, wheat, patent baker's grade	13 3	1 5	72 7	28
rye	6 8	0 9	78 7	29
Corn meal	9 2	1 9	75 4	28
Bread, average white	9.2	1 3	53 1	38
whole wheat	9.7	0 9	49 7	41
Macaroni	13.4	0 9	74 1	28
Oatmeal	16 1	7 2	67 5	25
Barley	8.5	1.1	77.8	28
Rice	8 0	0.3	79 0	29

TABLE XVIII

Composition of Some Vegetables and Legumes*

	Per Cent Protein (N × 6.25)	Per Cent Fat	Per Cent Carbohydrate	Portion in Grams Equivalent to 100 Calories
Asparagus, cooked	2.1	3 3	2.2	213
Beets, cooked	2 3	0 1	7.4	252
Cabbage	1.6	0 3	5 6	317
Celery	1 1	0 1	3 3	542
Lettuce	1 2	0 3	2 9	525
Onions, fresh	1 6	0 3	9 9	206
Potatoes, white, raw	2 2	0 1	18.4	120
sweet	1.8	0 7	27.4	81
Spinach, fresh	2 1	0 3	3 2	417
Squash	1 4	0 5	9 0	217
Tomatoes, fresh	0 9	0 4	3.9	438
Beans, string, fresh	2 3	0 3	7.4	241
baked, canned	6 9	2 5	19 6	78
Peas, canned	3 6	0 2	9 8	180
dried	24 6	1 0	62 0	28
green	7 0	0 5	16 9	100

* See also C. Chatfield and G. Adams, U. S. Dept. Agr. Circ. 146 (1931).

CHAPTER VI

CHEMISTRY OF ENZYMES

In the preceding pages we have described the composition and properties of the three great classes of foodstuffs. We are now about to follow their fate, chemically, after ingestion. We shall describe the changes they undergo during digestion and absorption, study the substances into which they are converted, and the metabolism of these substances in the various tissues of the organism. But these changes are mediated to a large extent, in all the tissues, by the presence of enzymes, and we must, therefore, turn to a brief study of the enzymes themselves.

A water solution of sucrose will remain indefinitely at room temperature without undergoing hydrolysis to fructose and glucose. If, however, to such a solution a 0.1 *N* concentration of hydrogen ion in the form of, say, hydrochloric acid, is added, the sucrose will be converted to a considerable extent within a few hours. The same result may be accomplished and, on the whole, within a much shorter time, by adding instead a filtered, concentrated extract of autolyzed yeast cells.

The action in the presence of the hydrogen ion, or of the autolyzed yeast extract, is spoken of as catalytic; that is, there is an increase, from a negligible to a very considerable level, in the rate at which the reaction proceeds to equilibrium. We may, therefore, define an enzyme as a catalyst, organic in nature, produced by living cells, but in its action independent of their presence. Further definition is, at present, empirical rather than fundamental, consisting in the determination of the properties characteristic of enzymes. Such properties will become clearer as we proceed. We shall also discuss later the question whether or not enzymes are perfect catalysts.

Preparation of Enzyme Extracts. Until recently enzymes have been known by the effects which they produced. If, for instance, a body fluid, body secretion or excretion, a plant juice, or a tissue extract is added to a solution of sodium-β-glycerophosphate and leads to the formation of inorganic phosphate, it is considered to be a preparation of phosphatase. This same preparation may, however, contain substances which affect the activity of the phosphatase, or it may even contain other enzymes acting on entirely different substrates. Certain procedures are, therefore, used to raise the concentration of the particular enzyme of interest, to remove substances depressing its activity, and finally to separate it from the other enzymes in the solution.

Most preparations of enzymes are obtained from cells or tissues. It is therefore first necessary to rupture the cell wall, then usually to extract the enzyme with some suitable solvent. Rupture may be accomplished by freezing and thawing, grinding the tissue with sand, maceration, or allowing the tissue enzymes themselves, usually the protein-splitting enzymes, to act upon the cell walls, thereby leading to their dissolution (autolysis). Any combination of these procedures may also be used. Sometimes preliminary drying of the tissue by extraction with an organic solvent reduces the tissue to an easily powdered substance and facilitates subsequent extraction.

The simplest solvent to use for extraction is water; many enzymes are very readily extracted in this way. Quite often, however, it is necessary to use solutions of various salts, of acid, or of alkali. Organic solvents such as acetone, glycerol, or mixtures of each with water are also utilized.

The above procedures may be briefly illustrated. Willstätter and Waldschmidt-Leitz [1] extracted the fat from pig pancreas with acetone and ether, then ground the defatted tissue with a water-glycerol mixture, thus obtaining lipase. Hudson [2] added toluene to pressed yeast; in several days the whole mass liquefied, and filtration yielded a strong preparation of invertase. Battelli and Stern [3] attempted to prepare succinoxidase by treating chopped muscle with water, but did not obtain an active extract. However, when the water was made slightly alkaline with disodium phosphate, the succinoxidase was easily extracted.

Purification of Enzyme Preparations. As with other substances of biological importance, a prime interest in the study of enzymes has been to isolate the various enzymes as chemical individuals. In recent years, this line of investigation has reached a high level with the crystallization of several of these substances. However, a good deal of work, yielding valuable information with regard both to composition and properties, has been and still is being carried out with preparations, purified to some extent, but not yet obtainable in crystalline form. It is therefore of importance to consider the general methods by means of which enzymes are prepared in concentrated solutions, free of major impurities, and usually also of other enzymes.

The particular scheme of purification and concentration of an enzyme extract depends upon the specific enzyme. There are, however, several general procedures which may be used in varying orders and combinations:

1. Dialysis, to rid the preparation of small-sized molecular impurities.

2. Precipitation, single, or repeated, by alcohol, by ammonium,

[1] *Z. physiol. Chem.*, **125**, 132 (1923).

[2] *J. Am. Chem. Soc.*, **30**, 1160 (1908).

[3] *Compt. rend. acad. Sci. Geneva*, **37**, 65 (1922); cited by J. B. S. Haldane, "Enzymes," pp. 168, 182 (1930).

sodium, or magnesium sulfates, or by addition of acid to a given *p*H, and re-solution of the precipitates.

3. Adsorption of the enzyme to certain materials, notably kaolin and alumina, and elution from these adsorption compounds by means of dilute solutions of ammonia, sodium carbonate, disodium phosphate, etc. Dialysis may be repeated at any stage to clear the preparation of substances introduced as reagents in the procedure—e.g., ammonium sulfate.

The above principles may be illustrated with a representative procedure by Willstätter and his coworkers,[4] in the case of invertase. A neutral chloroform-water or toluene-water autolysate of yeast was filtered, then precipitated with alcohol. The precipitate was extracted with *M* 50 acetic acid, and the resulting solution was treated with kaolin to adsorb the invertase. The enzyme was then eluted from the adsorption compound by means of dilute ammonia, sodium carbonate, or disodium phosphate. The resulting solution was dialyzed, made slightly acid, and treated with alumina. The alumina thus adsorbed the invertase; the invertase was freed again by means of disodium phosphate or arsenate. Such adsorption and elution was repeated one to three times and the final solution of enzyme was dialyzed. The enzyme became progressively more concentrated during the entire procedure. Thus, to take a representative result, 50 mg. of the original autolysate (determined as dry weight of the solution) required about 100 minutes to hydrolyze about 75 per cent of a standard, buffered solution of sucrose at 15.5° C. The same amount of the final product required only 0.1 to 0.2 minute. This, therefore, represented a 500–1000 fold purification of the autolysate.

Methods such as the above have been used to separate enzymes contained in a tissue extract. Willstätter and Waldschmidt-Leitz [1] found that, in pancreatic extracts containing lipase, amylase, and trypsin, the lipase was most readily adsorbed by alumina and kaolin and could thus be separated from the others. Treatment with kaolin in acid solution then resulted in the separation of trypsin from amylase.

Further details regarding the above methods for many other enzymes can be found in the comprehensive works of Oppenheimer.[5]

Crystallization of Enzymes. The purification of enzymes has reached its highest level of achievement in the crystallization of some of them. The methods used have been radically different from those

[4] R. Willstätter and K. Schneider, *Z. physiol. Chem.*, **142**, 257 (1924–25); R. Willstätter and F. Racke, *Ann.*, **425**, 1 (1921); **427**, 111 (1922); see especially K. Schneider in "Die Methodik der Fermente," by Oppenheimer and Pincussen, Leipzig, 1929.

[5] C. Oppenheimer, "Lehrbuch der Enzyme," Leipzig, 1927; "Die Fermente und Ihre Wirkungen," 5th ed., Leipzig, 1925; Supplement volume, Hague, 1935. A shorter book (in English) containing much information about the preparation and properties of enzymes is "Enzyme Chemistry" by H. Tauber, John Wiley & Sons, New York, 1937.

employed by Willstätter and his school. To date the following enzymes have been obtained in crystalline form:

Urease (Sumner, 1926).[6]
Pepsin (Northrop, 1930).[7]
Trypsin (Northrop and Kunitz, 1932).[8]
Chymotrypsin (Northrop and Kunitz, 1935).[9]
Warburg's respiratory ferment (Theorell, 1934).[10]
Carboxypeptidase (Anson, 1935).[11]
Catalase (Sumner and Dounce, 1937).[12]

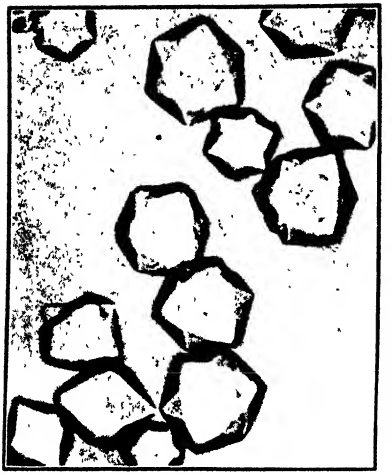
In addition, the proenzymes of pepsin, trypsin, chymotrypsin, and carboxypeptidase have also been prepared in crystalline form.

There is at present no general procedure for the crystallization of enzymes. However, the work that has been done indicates that, during the various procedures, the solutions of enzymes must be kept concentrated to prevent inactivation, and that the final induction of crystallization depends upon a gradual precipitation of the enzyme from its solution. The particular sequence of procedures used for a given enzyme depends upon the judicious observation and interpretation of its precipitating properties. Once crystals are obtained, the crystallization of other batches is considerably facilitated by inoculation. Several examples of the crystallization of enzymes are given below to illustrate the particular procedures followed. For fuller details and for the modifications finally adopted by the various investigators the student is referred to the original papers.

Urease. Sumner [6] obtained crystalline urease quite simply by stirring jack-bean meal with acetone and filtering the mixture overnight in the ice chest. Octahedral crystals appeared. The enzyme was recrystallized by dissolving these crystals in a small volume of acetone-water mixture and adding to an acetone-water mixture buffered at *p*H 6.1.

Pepsin. An entirely different procedure was followed in the preparation of pepsin. Pekelharing [13] in 1896 found that a precipitate showing considerable peptic activity appeared when an acid solution of the fundic portion of the gastric mucosa was dialyzed. Northrop [7] noted that this precipitate was granular, as if it were on the verge of crystallization. This precipitate dissolved when it was warmed in water to 45° C.; when the solution was allowed to cool slowly, crystals appeared. As a more regular procedure, Northrop precipitated the enzyme with magnesium

[6] *J. Biol. Chem.*, **69**, 435 (1926); **70**, 97 (1926).
[7] *J. Gen. Physiol.*, **13**, 739 (1930).
[8] *Ibid.*, **16**, 267 (1932). For a summary see J. H. Northrop, "Isolation and Properties of Pepsin and Trypsin," *Harvey Lectures*, **30**, 229 (1934–35).
[9] *J. Gen. Physiol.*, **18**, 433 (1934-35).
[10] *Biochem. Z.*, **272**, 155 (1934); **278**, 263 (1935).
[11] *Science*, **81**, 467 (1935); *J. Gen. Physiol.*, **20**, 663 (1936-37).
[12] *J. Biol. Chem.*, **121**, 417 (1937).
[13] *Z. physiol. Chem.*, **22**, 233 (1896).



Urease

FIG. 9.—Urease recrystallized from water. × 360. Courtesy of Professor James B. Sumner.

FIG. 10.—Pepsin. Courtesy of J. H. Northrop.

FIG. 11.—Trypsin. Courtesy of J. H. Northrop and M. Kunitz.

FIG. 12.—Catalase (cattle liver). Recrystallized prisms formed by chilling a strong alkaline phosphate solution. × 250. Courtesy of Professor J. B. Sumner and A. L. Dounce.

sulfate from an acidified, concentrated solution of a commercial preparation, then repeatedly dissolved and precipitated with sodium hydroxide and acid, respectively. Final crystallization was obtained by carefully dissolving the precipitate, warming the solution to 45°, then allowing it to cool slowly after inoculation with a crystal of pepsin.

Trypsin. In the case of trypsin, Northrop and Kunitz [8] made an extract of commercial pancreatin with one-quarter saturated ammonium sulfate. On addition of saturated ammonium sulfate to this extract, it was found that the most active precipitate appeared when the resultant concentration of ammonium sulfate was 0.6 *N*. This precipitate was separated and made up in a twentyfold volume of solution consisting of 0.25 saturated ammonium sulfate and 0.1 *M* acetate buffer of *p*H 4.0. Saturated ammonium sulfate was added cautiously at 25–30° C.; an amorphous precipitate was formed and filtered off. When the solution was allowed to stand small crystals of cubic form appeared. Northrop's final method was a modification of this, starting with fresh pancreas.

Chemical Nature of Enzymes. The first attempts to study the chemical nature of enzymes consisted in testing potent preparations directly for the presence of certain elements or of compounds. Thus Pekelharing [14] found his pepsin preparations to yield characteristic tests for protein; Sherman [15] obtained similar results for pancreatic and salivary amylases, and Euler and Josephson [16] found positive biuret, xanthoproteic, and ninhydrin reactions in active preparations of invertase.

The failure of agreement between two leading schools of investigators illustrates the approximate nature of this earlier work. Thus Euler and Josephson stated that there was proportionality between the activity and the nitrogen content of invertase preparations. Willstätter and his students failed to find such parallelism. Euler stressed the protein nature of the enzyme. Willstätter stated that he obtained invertase preparations which were free of protein, and others which were free of yeast gum. Euler believed that the protein was an important part of the enzyme molecule, determining its specificity as well as its activity. Willstätter looked upon the presence of protein as an accident; it was merely a colloidal carrier of a catalytically active compound; other substances such as peptides or gums could also play the rôle of carrier.[17]

The significance of such work was obscured by the fact that the chemical analyses which were being made of enzyme preparations were of the accompanying materials, as well as of the enzyme, and that these materials differed according to the method of preparation. Secondly, negative tests, such as Willstätter's failure to find protein, meant merely

[14] *Ibid.*, **35**, 8 (1902).

[15] *J. Am. Chem. Soc.*, **33**, 1195 (1911); **34**, 1104 (1912); **35**, 1790 (1913).

[16] *Ber.* **56B**, 1097 (1923); **57B**, 299 (1924); *Z. physiol. Chem.*, **133**, 279 (1924).

[17] *Z. physiol. Chem.*, **123**, 1 (1922); **147**, 248 (1925); **151**, 1 (1926); see also J. M. Nelson, *Chem. Rev.*, **12**, 1 (1933) for a discussion of the chemistry of invertase.

that protein was not present in sufficient concentration to give a typical reaction. Several years later Northrop,[18] for instance, showed that crystalline pepsin present in sufficient concentration to exhibit marked activity failed to react in that concentration with the usual protein reagents.

In recent years the determination of the chemical nature of enzymes has assumed two new paths. First, the isolation of several enzymes in crystalline form has permitted studies to determine the extent to which reaction with these compounds alters enzyme activity. Secondly, both in these crystalline enzymes and in partly purified enzyme preparations, the relation between certain chemical groupings in the enzyme and the activity has been studied by means of special reagents.

All the enzyme substances which have so far been isolated appear to be crystalline proteins or, at least, inseparably associated with them. Thus the crystalline urease obtained by Sumner gave all the protein tests; the Molisch reaction for carbohydrate was negative and the ash amounted to 1–2 per cent. The elementary analysis of a twice-recrystallized preparation was that of a protein: C, 51.6 per cent; H, 7.1; N, 16; S, 1.2. The substance was soluble in water except in the vicinity of the isoelectric point, where salt was required to keep it in solution. Sumner considered urease to be a globulin. Again, crystalline pepsin and trypsin show reactions and compositions typical of proteins. For instance, the elementary composition of pepsin has been found to be: C, 52.3; H, 6.66; N, 15.2; Cl, 0.23; S, 0.88; P, 0.078; ash, 0.40 per cent.

Upon the preparation of various crystalline proteins possessing enzymic properties a very crucial question suggested itself: Was the protein so prepared the enzyme itself, or was the enzyme activity due to some other substance which was adsorbed to the protein? The ordinary method for settling such a question would be to determine whether the physical properties and the chemical composition of the crystals remained constant after repeated crystallization. Though Northrop [18] found that seven recrystallizations of pepsin failed to alter significantly the proteolytic activity, optical rotation, or the percentage of nitrogen and phosphorus, there still remained the possibility that the crystals were a mixture of two substances present in amounts proportional to their solubilities. Moreover, Sørensen [19] had pointed out in 1926 that closely related proteins may be obtained in crystalline form as solid solutions, that such solid solutions are extremely difficult to separate by fractional crystallization, but that their existence may be detected by solubility measurements.

Northrop attacked the problem of the identity of the enzyme with the crystalline protein using a varied array of methods. He showed that the solubility of the crystalline protein in various salt solutions was independent of the amount of solid present, that the specific proteolytic

[18] *Biol. Rev.*, **10**, 263 (1935).

[19] S. P. L. Sørensen, "Proteins," Fleischmann & Co., New York, 1925.

activity and the specific optical activity for the material present in the solution and for the original material were the same. He found that the temperature coefficient for the inactivation of the enzyme was very high and the same as that for the denaturation of protein, that the conditions for inactivation by heat, or by alkali, and the conditions for reversal of such inactivation were the same for enzyme and protein. The value for the diffusion coefficient was the same whether measured by nitrogen content or by enzyme activity.

The conclusion seemed inescapable, then, that the enzyme activity was not due to a non-protein compound; that, if it were due to some other compound in solid solution with the protein isolated, the properties of this compound were those of a protein in every respect tested by Northrop.

We may conclude, on the basis of the studies just described, that those enzymes which so far have been obtained in crystalline form are proteins. Our next interest in the study of the chemical nature of enzymes is to find out something about the chemical groups or groupings in the enzyme molecule which are responsible for its characteristic activity.

In the case of pepsin it appears that acetylation of a primary amino group does not change the activity, whereas acetylation of other groups, especially the hydroxy group of tyrosine, results in decreasing the activity of the enzyme. Thus Herriott and Northrop [20] found, on acetylation of pepsin with ketene ($CH_2{=}C{=}O$), that they could isolate three crystalline compounds. The first derivative contained four acetyl groups attached to the four primary amino groups of the original pepsin; this compound had the same activity as the original pepsin. A second derivative contained seven acetyl groups, of which three acetyl groups were attached to the tyrosine phenol groups; this derivative had 60 per cent the activity of the original pepsin. A third derivative contained 18 to 20 acetyl groups per mole, 8 to 10 of which were attached to tyrosine phenol groups; this compound was 10 per cent as active as the original pepsin.

Another chemical grouping which appears to contribute crucially to the activity of some enzymes is the —SH— or sulfhydryl group. Perlzweig [21] noted that, when crystalline urease was partly inactivated by aeration, the nitroprusside test for thiol (—SH—) groups became weaker. Hellerman, Perkins and Clark [22] found that treatment of this enzyme with organic mercurials of the type R—Hg—X (where X=Cl or OH) abolished the activity, and subsequent treatment with hydrogen sulfide, hydrocyanic acid, or certain mercaptans restored the activity. Similarly the controlled use of certain oxidizing agents like iodine,

[20] *J. Gen. Physiol.*, **18**, 35 (1934); R. M. Herriott, *ibid.*, **19**, 283 (1935); J. H Northrop, *Physiol. Rev.*, **17**, 144 (1937).

[21] *Science*, **76**, 435 (1932).

[22] *Proc. Natl. Acad. Sci. U. S.*, **19**, 855 (1933); L. Hellerman, *Physiol. Rev.*, **17**, 454 (1937).

quinine, or aeration in the presence of cupric ion led to the inactivation of urease, and the addition of cysteine, or reduced glutathione (p. 303) reversed the inactivation. The proteinase constituent of the proteolytic enzyme, papain, behaved similarly in many respects. Hellerman and associates have therefore conceived the activity in these enzymes to be dependent on the intactness of the —SH— group; modification by oxidation to the —S—S— form, or combination with a metallic ion, results in inactivity; restoration of the activity is due to the reduction of the —S—S— group to —SH—, or to hydrolysis or decomposition of the metallomercaptide.

The rôle of metallic ions as apparent accelerators of enzyme action has long been recognized, as for example, the effect of magnesium on the activity of phosphatase.[23, 24] Catalase, peroxidase, and Warburg's respiration system (p. 307) contain iron, quite probably in the form of an iron-porphyrin complex.[25] Hellerman and Perkins [26] have recently suggested that some enzymes may be metal complexes. In the case of arginase, they found that the activity was not altered by mercaptide-forming organomercurials, nor by potent reducing agents; this militated against the sulfhydryl grouping being a determinative factor for this enzyme. On the other hand, ferrous, manganous, nickelous, and cobaltous ions restored the activity of the enzyme, after it had been suppressed with inhibiting oxidants.

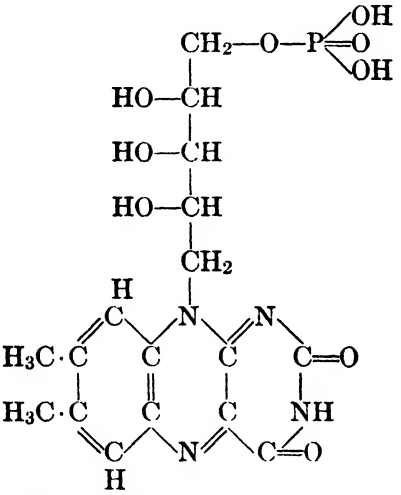
Further investigations into the chemical nature of enzymes have also revealed the possibility that the characteristic properties of a given enzyme may be due to the peculiar union between a protein component and another large chemical group, in much the same way, as we shall see in a later chapter, that the properties of hemoglobin are due to the combination of globin and heme. Such chemical groups have been termed *prosthetic* groups; their existence in certain enzymes has been definitely shown.

Catalase. When an acetone concentrate of catalase is added to acetone containing hydrochloric acid, a colorless protein precipitate is formed. The supernatant fluid is gray-blue in color, and yields, when the acetone is distilled off, a red microcrystalline precipitate. Stern,[27] in continuation of his own and Zeile's work,[28] has shown that this compound is an iron-porphyrin complex and in fact identical with the hemin obtained from hemoglobin (p. 244). The protein part of the enzyme complex seems, however, to differ from the globin of hemoglobin both in its isoelectric point (that of catalase is *p*H 5.2 to 5.5; of hemoglobin, about 7.0) and in its behavior towards certain precipitating agents.

[23] H. D. Jenner and H. D. Kay, *J. Biol. Chem.*, **93**, 733 (1931).
[24] O. Bodansky, *ibid.*, **114**, 273; **115**, 101 (1936).
[25] D. Keilin, *Ergeb. Enzymforsch.*, **2**, 239 (1935).
[26] *J. Biol. Chem.*, **112**, 175 (1935).
[27] *Z. physiol. Chem.*, **208**, 86 (1932); **215**. 35 (1933); *J. Biol. Chem.*, **112**, 661 (1935).
[28] *Z. physiol. Chem.*, **195**, 39 (1931); Zeile and Hellström, *ibid.*, **192**, 171 (1930).

Evidence has also been presented for the existence of an iron-porphyrin complex in peroxidase.[29]

"Yellow Enzyme." Decisive advances have also been made with regard to the chemical nature of the "yellow enzyme." This enzyme is part of a system which is necessary for the oxidation of hexosemonophosphoric acid (p. 310). When this enzyme is dialyzed against dilute hydrochloric acid, it splits into two portions, a dye and a protein, each inactive. The dye was recognized as a flavin by Warburg and Christian[30] and by others; it was finally synthesized by Kuhn, Rudy, and Weygand [31] in 1936. It is lactoflavin-5′-phosphoric acid, or 6,7-dimethyl-9-*dl*-ribitylisoalloxazine-5′-phosphoric acid. The structural formula is given below. It is to be noted that lactoflavin is a component of the vitamin B_2 complex (p. 591).



6,7-Dimethyl-9-*d*-ribitylisoalloxazine-5′-phosphoric acid (lactoflavin-5′-phosphoric acid)

Kuhn and Rudy [32] have combined lactoflavin-5′-phosphoric acid with the protein isolated from the yellow enzyme by Theorell,[10] thus reforming the catalytically active flavoprotein. Kuhn and his co-workers have also shown that the union of dimethyl-9-araboflavinphosphoric acid and Theorell's protein forms a catalytically active substance.

CLASSIFICATION OF ENZYMES

Enzymes are at present classified on the basis of the reactions which they catalyze. Such classification is, therefore, arbitrary to a considerable degree. First, reactions are themselves classified differently by

[29] D. B. Hand, *Ergeb. Enzymforsch.*, **2**, 272 (1933).
[30] *Biochem. Z.*, **266**, 377 (1933).
[31] *Ber.*, **69**, 1543 (1937).
[32] *Ibid.*, **69**, 1974, 2034 (1936).

different investigators. Thus, Haldane [33] considers, as one group, the enzymes hydrolizing the C—N—linkage; accordingly urease which splits urea into ammonia and carbon dioxide is classed with pepsin which acts on proteins to increase the amino and carboxyl groups. Sumner,[34] on the other hand, groups such enzymes as urease, arginase, and desamidase as the *aminoacylases*; he places pepsin, trypsin, the various peptidases, etc., into the group of *proteolytic* enzymes. Secondly, the question of specificity, which we shall discuss in the next section, raises the possibility of subclassification within a given group. Finally, as we shall see in a later chapter, the enzymes involved in oxidation-reduction processes act in groups, as systems of enzymes. The elucidation of the rôle of each of the enzymes in such a system has proceeded significantly only within the past few years.

Keeping the above reservations in mind, we may, on the whole, accept as our basis of classification Haldane's scheme, illustrating each group with several examples of immediate interest in our study of digestion and metabolism. For more nearly complete lists, the student should consult the works of Haldane [33] and Oppenheimer.[6] It should be noted that usually, *though not always*, the name of the enzyme indicates the name of the substance acted upon (*substrate*) and has the suffix *-ase*.

Group I. Enzymes Hydrolyzing Esters. In this group we have such examples as the *pancreatic and gastric lipases* which hydrolyze the glycerides of the higher fatty acids (the fats) more readily than they do the monoesters of the lower fatty acids. The reverse is true of *liver lipase*, which may therefore more correctly be called an *esterase*.

The *phosphatases*, present in yeast, kidney, intestine, bone, and other animal tissues, hydrolyze the linkage between phosphoric acid and the OH group in an enormous variety of compounds; phospholipids, phosphoglyceric acids, phosphoric esters of carbohydrates, etc. The significance of this enzyme or group of enzymes in bone formation and in general metabolism has received close attention in recent years.

Chlorophyllase occurs in green leaves and hydrolyzes chlorophyll a to chlorophylide a and phytol. *Choline esterase*, found in heart muscle, intestinal mucosa, brain, etc., hydrolyzes esters of choline and betaine.[35] Since acetylcholine is a physiologically important substance, this enzyme, the existence of which was suspected by Dale [36] in 1915, has received considerable attention during recent years.

Group II. Carbohydrases; Enzymes Hydrolyzing Osides and Polysaccharides. *Amylase* hydrolyzes starch to maltose. Kuhn [37] has divided the various amylases into the α- and the β-types, according

[33] J. B. S. Haldane, "Enzymes," Longmans, Green & Co., 1930.
[34] *Ann. Rev. Biochem.*, **4**, 37 (1935).
[35] E. Kahane and J. Levy, *Bull. soc. chim. biol.*, **18**, 505, 529 (1936).
[36] *J. Pharmacol.*, **6**, 147 (1914–15).
[37] *Ann.*, **443**, 1 (1925).

to whether α-maltose or β-maltose is produced. The better-known amylases are the salivary (*ptyalin*), pancreatic (*amylopsin*), liver, and, among the plants, *malt amylase.*

Sucrase (*invertase, saccharase*) is found in the intestinal mucosa, in the intestinal juice, and in yeast; it hydrolyzes sucrose to glucose and fructose.

Maltase (α-*d*-glucosidase) hydrolyzes maltose and α-*d*-glucosides, as the name suggests; it is found in almost all plant and animal tissues.

Lactase (β-*d*-galactosidase) is found in the intestinal mucosa and juice and hydrolyzes β-galactosides, including lactose and melibiose.

Emulsin, found in bitter almonds, hydrolyzes β-glucosides. Other enzymes belonging to the group of carbohydrases are *cellulase, inulase, trehalase.*

Group III. Enzymes Hydrolyzing the C-N Linkage. *Pepsin,* found in the gastric mucosa (and juice); *trypsin* (pancreatic juice); *kathepsin,* found in the tissues generally, hydrolyze proteins to proteoses and peptones.

Bromelin (pineapple) and *papain,* found in the pawpaw, have, similarly, a proteolytic function.

The lower-split protein products are hydrolyzed by the various *peptidases* into amino acids.

Rennin hydrolyzes casein to form paracasein, which precipitates as an insoluble calcium salt.

In the group of enzymes which hydrolyze the C-N linkage there are several which have been called *amidases* or *aminocyclases* (also *deamidases, deaminases*). These include *urease* (urea to carbon dioxide and ammonia); *arginase* (arginine to urea and ornithine, see p. 411); *asparaginase* (asparagine to ammonia and aspartic acid); and the various enzymes which deaminate the purines (*guanase, adenase,* etc.).

Enzymes Involved in Oxidation-Reduction Processes. Within the past few years the concept that biological oxidation processes are systems of linked oxidation-reduction reactions has gained additional support. It has been shown, moreover, that prosthetic groups (p. 134) of the enzymes themselves, or of the coenzymes, contain chemical groupings which are capable of existing in a reduced or oxidized stage. In Chapter X we shall enter into this question more thoroughly. Though the understanding of the mechanisms involved is at present still not completely clear, we may nevertheless make a tentative and approximate classification at this point.[38]

There is, first, a group of enzymes, termed *dehydrogenases* (citric, lactic, etc.). Some investigators have conceived the function of these enzymes to be an activation of substrate, so that the substrate and a second substance constitute a reductant-oxidant pair. Several dehy-

[38] Classification is difficult because, as will be shown later in Chapter X, groups of enzymes appear to be involved in the oxidation of various metabolites.

drogenases have been shown to consist of two parts: a coenzyme containing an oxidizable-reducible pyridine nucleus and a protein, known variously as "*zwischenferment*" and *apodehydrogenase.*

Oxidases represent the group of enzymes in the presence of which the reduced form of a cell constituent will be reoxidized.

Catalase liberates oxygen from hydrogen peroxide and thus serves the cell economy by producing a source of molecular oxygen from the hydrogen peroxide presumably formed in the system of cell oxidations.

Peroxidase leads to the oxidation of certain substances, like pyrogallic acid, in the presence of hydrogen peroxide.

Miscellaneous Enzymes. *Fumarase* and *glyoxalase* will insert water without subsequent hydrolysis. Fumarase converts fumaric acid into *l*-malic acid; glyoxalase acts on methylglyoxal and other substituted glyoxals to form the corresponding glycollic acids.

Aspartase removes ammonia without hydrolysis. It acts on aspartic acid to form fumaric acid.

Carboxylase leads to the liberation of carbon dioxide from the carboxyl group. *Carbonic anhydrase* liberates carbon dioxide from the carbonate ion (p. 256).

Specificity. We have already had sufficient opportunity to note that there exists a relation between a given enzyme preparation and the chemical structure of the substances which react in its presence. This relation is known as *specificity.* In fact, the system of classification which we have just discussed implies, in a broad way, the existence of such a relation.[39]

However, specificity has been demonstrated in much greater detail in several instances. For example, the action of emulsin is influenced in a striking manner by the group attached to β-*d*-glucose. The rates of hydrolysis for the following glucosides increase in the order: methyl-β-glucoside, glucose-β-glucoside, phenyl-β-glucoside, saligenin-β-glucoside.

In general, the action of enzymes is limited not so much to certain substances as to specific atomic groups or linkages in the molecule. Thus, lipases act on a variety of esters, maltase acts not only on maltose, but also on other α-glucosides, whereas emulsin hydrolyzes various

[39] In enzymic reactions, specificity may be either with respect to the substance attacked as indicated above or the type of products formed. Thus both yeast saccharase and emulsin hydrolyze the trisaccharide, raffinose. But the first forms fructose and melibiose; the latter forms sucrose and galactose. Glucose may undergo several types of fermentations, each being caused by a specific enzyme. The substrate is the same, but the products, lactic acid, alcohol, etc., are different. On the other hand, the reaction products may be identical but the reaction path different. Both the saccharase of yeast and that of *Aspergillus oryzae* invert cane sugar to glucose and fructose. But according to Kuhn (*Z. physiol. Chem.*, **129**, 57 [1923]), the former does so by attaching itself to the fructose component of cane sugar, whereas the enzyme of the mold-fungus is supposed to attach itself to the glucose part of the molecule.

β-glucosides, although, to be sure, at different rates. The action of emulsin is also influenced by the constitution of the sugar component, as shown by the data in Table XIX for a number of phenyl derivatives.

TABLE XIX

COMPARISON OF RATES OF HYDROLYSIS BY EMULSIN WITH VARIATION IN STRUCTURE OF THE SUBSTRATE (AFTER HELFERICH*)

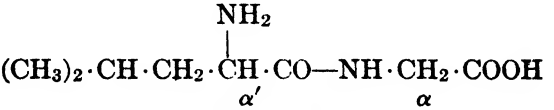
Substrate	Time for 50 Per Cent Change, Minutes
Phenyl-β-*d*-glucoside	6.9
Phenyl-β-*d*-maltoside	13
Phenyl-β-*d*-isorhamnoside	20
Phenyl-β-*d*-galactoside	57
Phenyl-β-*d*-glucoside-6-bromhydrin	670
Phenyl-β-*d*-xyloside	1000

* B Helferich, *Ergeb. Enzymforsch.*, **2**, 74 (1933).

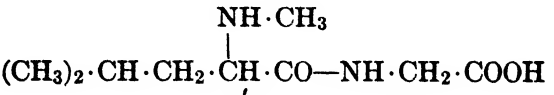
The specificity of the enzymic hydrolysis of various peptides has been investigated in great detail. Bergmann,[40] for example, postulates the following conditions for the action of *dipeptidase*:

1. The substrate must contain a peptide linkage, a free carboxyl group, and a free amino group.
2. The carboxyl group must be on the carbon atom next to the peptide nitrogen atom.
3. The amino group must be on the carbon atom next to the peptide carbonyl.
4. A peptide hydrogen atom is necessary.
5. Two hydrogen atoms must be in an α, α′-position.

For example, a given enzyme preparation splits 70 per cent of *dl-leucylglycine* in 1 hour, 86 per cent in 2 hours, but only 4 per cent of *n*-methyl-*dl*-leucylglycine in 2 hours.



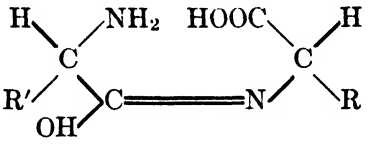
dl-Leucylglycine



n-Methyl-*dl*-leucylglycine

[40] M. Bergmann, *et al.*, *J. Biol. Chem.*, **109**, 325 (1935); *Harvey Lectures*, **31**, 37 (1935–36).

It is possible to explain specificity either by assuming that a given enzyme preparation consists of a mixture of several enzymes, each having a definite, specific action on one substrate, or by assuming that the enzymic action depends upon the extent to which the enzyme can, structurally, fit with or approach to the substrate. The latter explanation is the one more commonly held. Bergmann and his collaborators, for instance, conceive the mechanism of dipeptidase action to be as follows. Under the influence of the enzyme, the amide form of the peptide is changed to the imide variation. The configuration of the dipeptide is then as pictured below. It is like an hexagon with the six corners lying in one plane; the 2H atoms and the R and R′ groups lie in a *cis* position with respect to each other.



The enzyme approaches the hexagon on the side of the H atoms, which, being of small molecular volume, do not hinder such approach. On the other hand, when a methyl group is substituted for one of the H atoms, approach is not so easy and union not so complete; the rate of hydrolysis is therefore much slower.

Bergmann has investigated the action on various types of peptides, assuming in general the above mechanism and attempting to isolate different peptidases to account for different groups of actions. Helferich (See note to Table XIX), in his work on emulsin, also brings forward evidence that one enzyme is concerned. It is important, however, to recall that Northrop's exhaustive work with the crystalline pepsin does not exclude the possibility that what we consider as one enzyme may be a mixture of crystalline proteins alike in almost all properties and actions, but perhaps differing slightly in the way each acts on similar substrates.

Enzyme Models. We have previously noted the progress which has been made towards determining the structure of enzymes. In recent years, attention has also been given to the converse problem: Are there substances of known chemical structure which simulate enzymic activity? Kisch [41] and his co-workers have studied the oxidative deamination of amino acids by means of quinone and quinone derivatives. Employing the catalyst in low concentrations, about 0.001 *M*, they have found that the action proceeds optimally at certain *p*H levels—about *p*H 10. Oxidative deamination of glycine or serine is accompanied by formation of equivalent amounts of carbon dioxide and

[41] Among many articles on the subject, the following may be noted: *Biochem. Z.*, **242**, 1 (1931); **254**, 148 (1932); **259**, 455 (1933); **268**, 158 (1934).

ammonia. However, decarboxylation is also noticeable under anoxidative conditions when hydrogen acceptors are present.

Animal charcoal has been reported to function as a catalyst in the reaction: Succinic acid + methylene blue = fumaric acid + leucomethylene blue.[42]

The effect of diethylaminocellulose as a catalyst in the formation of optically active oxynitriles from benzaldehyde and cyanide has been studied by Bredig and associates.[43]

Enzymes as Catalysts. It was stated at the beginning of the chapter that an enzyme is a catalyst. Its function as such is to increase the rate with which a reaction proceeds to equilibrium, but not to alter the equilibrium itself. Moreover, if an enzyme operates as a perfect catalyst, then its concentration at the beginning of the reaction, E_a, should be equal to its concentration at the end, E_b. The equilibrium constant, K, where the reaction is:

$$A + X + E_a \rightleftharpoons B + Y + E_b$$

and

$$K = \frac{(B)(Y)(E_b)}{(A)(X)(E_a)}$$

should be independent of the enzyme concentration and should, as stated above, be the same whether the enzyme is present or not.

The precise determination of K from measurement of the concentration of reactants is not easily accomplished because of experimental difficulties. Borsook [44] has therefore advised the use of other data (thermal, solubility, ionization) in the calculation of the free energy change or equilibrium constant. He and his co-workers have considered three enzyme reactions:

(1) $\text{Succinate}^- \rightleftharpoons \text{fumarate}^- + 2H^+ + 2e$
(2) $l\text{-Aspartic acid} \rightleftharpoons \text{fumaric acid} + NH_3$
(3) $H_2 \rightleftharpoons 2H^+ + 2e$

The equilibrium constant calculated from data obtained without the use of the enzyme agreed with that when the enzyme was present. In these cases, then, it would seem that the enzyme behaves as a perfect catalyst.

FACTORS CONTROLLING VELOCITY OF ENZYME REACTION

The study of the factors which influence the velocity of enzymic reaction contributes to our knowledge of the nature of the enzymes. In addition, as will be seen, knowledge of certain basic factors is a neces-

[42] B. Tamamuchi and H. Umezawa, *Acta Phytochim.* (Japan), **8**, 221 (1935); cited in *Ann. Rev. Biochem.*, **5**, 54 (1936).
[43] *Biochem. Z.*, **282**, 88 (1935).
[44] *Ergeb. Enzymforsch.*, **4**, 1 (1935).

sary preliminary to the evaluation of other factors, or to the study of the rôle of the enzyme in certain physiological processes.

In the following pages we shall consider these factors:

1. Concentration of substrate.
2. Concentration of enzyme.
3. Hydrogen-ion concentration.
4. Temperature.
5. Oxidation-reduction state of enzyme.
6. Inactivation of enzyme.
7. Products of reaction.
8. Activators.
9. Physical agents: light, radiations, etc.

Concentration of Substrate. In 1913 Michaelis and Menten [45] determined with precision the rate at which different concentrations of sucrose began to hydrolyze in the presence of a given concentration of invertase; all other conditions were, of course, held constant. The results are given in Table XX.

TABLE XX

EFFECT OF SUCROSE CONCENTRATION ON RATE OF HYDROLYSIS BY INVERTASE AT BEGINNING OF THE REACTION (AFTER MICHAELIS AND MENTEN[45] AND NELSON AND LARSON)

Initial Concentration of Sucrose		Rate of Hydrolysis as Measured by Change in Optical Rotation, Degrees Change per Minute	
Grams per 100 cc.	Mols per Liter	Experimental	Calculated*
0 263	0 0077	0 027	0.022
0 527	0 0154	0 035	0 038
1.05	0 0308	0 050	0 048
1.64	0 0480	0 058	0 055
3.28	0 0960	0 068	0 064
6.56	0 1920	0 075	0 069
13 12	0 3850	0 075	0 072
26 24	0 7700	0 063	0 073

* The values in this column have been calculated by J M. Nelson and H. W. Larson, *J. Biol. Chem.*, **73**, 223 (1927). They are those values which would be obtained from the relation given on p. 143.

According to the third column in Table XX, we see that, in the region up to a concentration of about 5 per cent sucrose, the initial rate of hydrolysis increases as the initial concentration of substrate increases. In the region from about 5 to 13 grams per 100 cc., the velocity remains

[45] *Biochem. Z.*, **49**, 333 (1913).

constant. Beyond that, the initial rate decreases. Such results are typical of those found for many enzymes.

What is the meaning of these results? Though there was evidence to show that enzymes might be colloidal, Michaelis and co-workers assumed that, in the usual experimental conditions, the reaction proceeded as in a homogeneous system. Michaelis and Menten, therefore, conceived the idea that sucrose and invertase formed an intermediate compound, that the rate of reaction was, in accordance with the mass law, proportional to the concentration of this compound, and that, further, this intermediate compound decomposed into glucose and fructose, the products of the reaction, and invertase. Such assumptions led to the expression:

$$\text{Rate of hydrolysis} = \frac{1}{\text{constant}} \times \frac{\text{concentration of sucrose}}{\text{concentration of sucrose} + K}$$

where K is the dissociation constant of the supposed intermediate compound and can be determined from the experimental results.

If we now turn to the fourth column of Table XX, we shall see the extent to which results calculated from the above expression actually agree with the results obtained experimentally. We see that this agreement is incomplete, especially at the higher concentrations of sucrose. Michaelis explained some of this deviation by stating that, as for other reactions, the mass law could not be expected to hold rigidly in concentrated solutions.

Because it was clear in conception and agreed well with the current theories of chemical reaction, Michaelis' formulation was widely accepted. The dissociation constant of the supposed intermediate compound was calculated for a wide variety of enzymes and under different conditions by many subsequent investigators.

It must be emphasized, however, that if we make the assumption that the reaction is heterogeneous instead of homogeneous, we obtain, following Langmuir's derivation,[46] the following expression:

$$\text{Rate of hydrolysis} = \text{constant} \times \frac{\text{concentration of sucrose}}{1/b + \text{concentration of sucrose}}$$

where b is the ratio of the constant describing the rate at which the molecules of sucrose strike the surfaces of the enzyme particle to the constant describing the rate at which they leave. As Hitchcock[47] first pointed out, the above formula is of precisely the same mathematical form as the equation of Michaelis.

From a logical point of view, therefore, the use of either the expression of Michaelis, or that of Langmuir, is not decisive in determining whether enzyme reactions proceed in homogeneous or in heterogeneous

[46] *J. Am. Chem. Soc.*, **38**, 221 (1916).
[47] *Ibid.*, **48**, 2870 (1926)

systems, whether the rate of reaction is dependent on the concentration of an intermediate compound, in Michaelis' sense, or whether it is proportional to the number of molecules of substrate leaving the surface of colloidal particles of enzyme. Stern [48] has recently submitted spectroscopic evidence for the formation of an intermediate compound during the action of catalase on monoethyl peroxide.

Concentration of the Enzyme. The rate of an enzymic reaction is directly proportional to the concentration of enzyme, within the rather wide variation of the latter that has so far been studied. Sometimes this direct proportionality between concentration and reaction rate is not apparent. It can be shown, however, in these few instances, that this failure in proportionality is due either (*a*) to the incorrect use of a measure of the reaction rate, or (*b*) to the presence of impurities which combine with the enzyme and retard its activity.[49]

In Table XXI is shown the reaction rate, expressed as the reciprocal of the time necessary to effect a 10 per cent change in the substrate, at different concentrations of a purified pepsin preparation. It may be noted that the ratio of the reaction velocity ($1/T$) to that of the concentration of enzyme (E) is constant as the concentration of enzyme is varied; in other words, the reaction velocity is directly proportional to the enzyme concentration. Similar results have been obtained for invertase, phosphatase, and many other enzymes.

TABLE XXI

DIRECT PROPORTIONALITY BETWEEN REACTION VELOCITY AND CONCENTRATION OF ENZYME IN A PURIFIED PREPARATION OF PEPSIN (AFTER NORTHROP[50])

E Relative Pepsin Concentration	$1/T$ Reciprocal of Time in Hours Necessary for 10 Per Cent Change in Albumin	$1/TE$ Ratio of Reaction Velocity to Concentration of Pepsin
12 5	0.59	0.047
25 0	1.20	0.048
50 0	2.50	0 050
66.0	3.23	0 049
100.0	5 00	0.050

In Table XXII are shown the rates of reaction at different concentrations of an unpurified preparation of pepsin. As the concentration of enzyme is increased, the ratio of the reaction velocity is less than that called for by a direct proportionality between it and the concentration

[48] *J. Biol. Chem.*, **114**, 473 (1936).

[49] For a fuller discussion of these points and of the measures of reaction velocity used in the study of biochemical reactions, see O. Bodansky, *Science*, **86**, 52 (1937), and *J. Biol. Chem.*, **120**, 555 (1937).

of enzyme. That this deviation from proportionality is due to products of protein digestion (peptones, etc.) present as impurities in combination with the pepsin was demonstrated by Northrop.[50]

TABLE XXII

RELATION BETWEEN REACTION VELOCITY AND CONCENTRATION OF ENZYME IN AN UNPURIFIED PREPARATION OF PEPSIN (AFTER NORTHROP)

E Relative Pepsin Concentration	$1/T$ Reciprocal of Time in Hours Necessary for a 10 Per Cent Change in Albumin	$1/TE$ Ratio of Reaction Velocity to Concentration of Pepsin
6.25	1 39	0.222
12.50	2 35	0.188
25.0	3.81	0 152
50.0	6 39	0.128
100 0	9.60	0 096

That impurities present as products of proteolytic autolysis may retard the action of the enzyme and so mask the relation of direct proportionality between reaction velocity and concentration of the enzyme has also been demonstrated for phosphatase.

Hydrogen-ion Concentration. The activity of an enzyme depends upon the hydrogen-ion concentration at which the reaction takes place. The influence of this factor may be of two kinds. First, at certain hydrogen-ion concentrations, the enzyme may undergo destruction while it acts on the substrate. Secondly, even though there is no destruction of enzyme, the rate at which it acts depends on the hydrogen-ion concentration of the solution, and there is a certain hydrogen-ion concentration at which the reaction velocity is optimal.

The destruction of enzyme at unfavorable hydrogen-ion concentrations may be demonstrated by determining the *p*H for optimal activity, then incubating a portion of the enzyme preparation at unfavorable *p*H for varying lengths of time, restoring the reaction to optimum *p*H and redetermining the activity of a sample. Fig. 13 is an illustration of such inactivation. It shows the percentage of active trypsin remaining after 30 minutes' incubation at 38° at various *p*H values. It also shows the effect of an inhibiting solution, consisting of the products of tryptic digestion, on such inactivation.

The activity of the enzyme at *p*H levels where there is no destruction is shown typically in Fig. 14, based on the experimental data of Michaelis and Davidsohn [51] on yeast invertase. We note that at *p*H 4.4 to 4.6

[50] *J. Gen. Physiol.*, **2**, 470 (1920).
[51] *Biochem. Z.*, **35**, 386 (1911).

the activity is optimal; on either side of this region, the activity decreases.

Several explanations have been offered to account quantitatively for this phenomenon. Michaelis and his co-workers considered invertase to

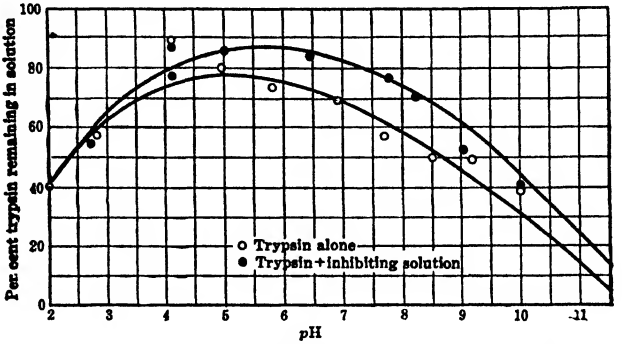


FIG. 13.—Inactivation of trypsin in relation to *p*H and the presence of inhibiting substances (products of tryptic digestion). Showing per cent of trypsin remaining after 30 minutes incubation at 38° C. After J. H. Northrop, *J. Gen. Physiol.*, **4**, 261 (1922).

be amphoteric, and its activity to be dependent on the proportion of unionized molecules. According to this concept, therefore, the activity should be optimal when the invertase is undissociated as it presumably is at *p*H 4.5. The activity should decrease when the invertase is ionized, as it is assumed to be in the alkaline region, *p*H 4.5 to 8.0. The *p*H-activity would thus resemble the "titration curve" of an acid. According to this concept and also to the one that the reaction rate is proportional to the concentration of an intermediate enzyme-substrate compound, the relation between *p*H and activity should be different at different sucrose concentrations. Michaelis and Rothstein,[52] however, did not find this to be so.

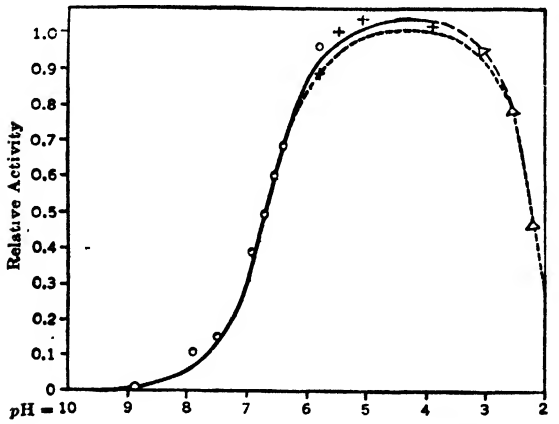


FIG. 14.—Activity *p*H curve for invertase (after Michaelis and Davidsohn).

Northrop,[53] on the basis of his work with pepsin and trypsin, offered

[52] *Ibid.*, **110**, 217 (1920).

[53] *J. Gen. Physiol.*, **5**, 263 (1923).

a different explanation for the effect of *p*H. He believed that the determining factor in the rate of digestion was the proportion of ionized protein, and that the *p*H affected this rather than the enzyme. This is illustrated in Fig. 15. We see that the curve (dotted) representing the activity of pepsin and trypsin as a function of *p*H when casein, for example, is used as substrate, is of practically the same form as the curve (plain line) representing the degree of ionization of the casein.

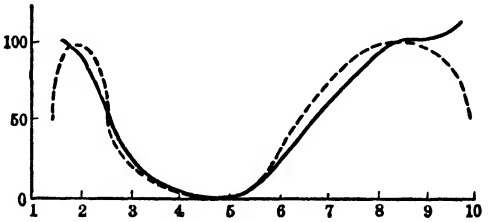


FIG. 15.—Showing the relation of the activity of pepsin and trypsin to the ionization of protein. Plain line, titration curve of casein; dotted line, rate of digestion (pepsin on left, trypsin on right). After J. H. Northrop, *J. Gen. Physiol.*, **5**, 263 (1922).

Kuhn [54] and Abderhalden [55] have explained the influence of the H-ion concentration as being due to its effect on the decomposition velocity of the enzyme-substrate combination.

However, whatever may be the explanation of the variation in enzyme activity with *p*H, the determination of the activity at optimal *p*H is a fundamental characterization of an enzyme preparation. It is, moreover, an important and simplifying preliminary to the study of the effect of other factors or agents on the activity of the enzyme. In Table XXIII are given the optimal *p*H values for the action of some of the more common enzymes.

TABLE XXIII

Enzyme	Optimal *p*H	Investigator
Yeast invertase	4 5–5 0	Nelson and Bloomfield*
"Alkaline" phosphatase	9 0–9 2	Kay†
"Acid" phosphatase	5 2–6 2	Kutscher and Wörner‡
Pancreatic lipase	7 0	Platt and Dawson§
Intestinal maltase (man)	6 1	Wigglesworth ‖
Pancreatic amylase	6 1	Myrbäck¶
Gastric pepsin	1 5–2 5	Northrop**
Pancreatic trypsin	8–11	Northrop**

* *J. Am. Chem. Soc.*, **46**, 1025 (1924). † *Biochem. J.*, **22**, 855 (1928). ‡ *Z. physiol. Chem.*, **239**, 109 (1936). § *Biochem. J.*, **19**, 860 (1925). ‖ *Ibid.*, **21**, 797 (1927). ¶ *Z. physiol Chem.*, **159**, 1 (1926). ** *J. Gen. Physiol.*, **5**, 263 (1922).

Effect of Temperature. A rise in temperature increases the velocity of a chemical reaction. Usually a rise of 10° results in a twofold to

[54] *Naturwissenschaften*, **11**, 732 (1923).
[55] *Proc. XIII Intern. Physiol. Cong., Am. J. Physiol.*, **90**, 258 (1929).

fourfold increase in the rate. The ratio of the velocities for such a temperature interval is known as the *temperature coefficient.*

However, a much more general and significant manner of expressing the effect of temperature is by means of the Arrhenius expression:

$$\frac{dlnk}{dT} = \frac{E}{RT^2} \tag{1}$$

where k is the reaction constant describing the reaction velocity at a given absolute temperature, T; R is the gas constant (1.98 calories per degree); and E is the constant known as the *critical increment,* or the apparent energy of activation. The above equation may also be expressed in an integral form (definite integral between two temperatures) as:

$$E = \frac{R(lnk_2 - lnk_1)}{1/T_1 - 1/T_2} \tag{2}$$

With enzymes, increasing the temperature increases the rate at which the reaction occurs. However, at a certain temperature, usually about 40°, the enzyme also begins to be destroyed. The apparent reaction velocity then becomes the resultant of these two opposing tendencies; the increase in velocity due to increased temperature, and the decrease in velocity due to the destruction of some enzyme. When the second of these processes gains ascendancy the observed reaction velocity begins to decrease. The temperature at which *optimal* action occurs is known as the *optimal temperature.*

The value of the temperature coefficient, or of the critical increment, has been determined for many chemical reactions by means of the reaction constant as a measure of reaction velocity. For enzymic reactions, values have not, on the whole, been determined with comparable accuracy. In Table XXIV are listed the temperature coefficients and critical increments which have been calculated for several enzymes by different investigators.[56]

A catalyst usually lowers the value of the critical increment for a given reaction. Thus the critical increment for the decomposition of hydrogen peroxide in aqueous solution is 18,000 calories; in the presence of the catalyst, iodide ion, it is 13,500 calories; and in the presence of colloidal platinum it is 11,700 calories.

If the catalyst is an enzyme, the lowering is much greater. Thus in the hydrolysis of sucrose in aqueous solution, when hydrogen ion is the catalyst, E (the critical increment or energy of activation) is 25,560 calories; in the presence of invertase, it is, as shown in Table XXIV, about 9000 calories.[56]

[56] For a fuller discussion consult A. E. Moelwyn-Hughes, "Kinetics of Reactions in Solution," p. 225, 1933; see also J. B. S. Haldane, "Enzymes," p. 66, 1930.

TABLE XXIV

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

Enzyme	Temperature	Temperature Coefficient	Critical Increment in Calories
Urease	20°–30°	1.81	
	30°–40°	1.90	
Malt amylase	20°–30°	1.96	12,100
	30°–40°	1.65	9,670
Invertase	0°–20°	....	10,500
	20°–52°	....	8,800
Invertase	15°–25°	1.76	8,700
	25°–35°	1.62	
Peroxidase	5°–15°	2 0	
	15°–25°	2 0	

Effect of State of Oxidation-Reduction. We have already seen (p. 134) that, in certain enzymes, the reduced form is active, the oxidized is inactive, and the two form, under certain conditions, a reversible system. According to Hellerman,[22] this may be pictured

$$\underset{\text{(Active)}}{2\,\text{En—SH}} \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} \underset{\text{(Inactive)}}{\text{En—S—S—H}} + 2(\text{H})$$

It is thus possible that a given preparation of such an enzyme be a mixture of the inactive oxidized form and the active reduced form, as, for example, a urease preparation that has inadvertently been aerated in the presence of cupric ion. In evaluating the concentration of the total enzyme in such a preparation, the oxidation-reduction state would have to be defined, or the activity determined, at a selected, standard state.

It is also possible to conceive that the activity of an enzyme within the cell is dependent on its oxidation-reduction state, and, therefore, on any factor in the cell, such as *p*H, oxidation-reduction intensity level, etc., which influences this state. At present, little is known of such relations *in vivo*.

Inactivation of Enzyme. During the course of making an enzyme preparation, it is often noted that there is, with a given procedure, a rather acute loss in the activity of the enzyme. Similar losses are noted when an enzyme solution is allowed to stand at room temperature. We have already seen that for certain enzymes such inactivation is probably due to the oxidation to an inactive form. However, a major source of inactivation appears to be the denaturation of the enzyme protein.

This type of inactivation has been well demonstrated for pepsin and trypsin by Northrop.[57] In general, the inactivation of an enzyme in solution depends on the purity of the preparation, its concentration, the temperature and the *p*H of the solution, and the length of time during which the enzyme is kept at the given conditions. Such inactivation may be reversible or irreversible—that is, after the enzyme has been subjected to certain conditions, it is inactive, but may regain its activity, either completely or in part, by being subjected to another set of conditions. If such conditions for restoring the activity have not been found, the inactivation is termed irreversible.

The purity of the enzyme preparation affects the inactivation. Thus a crude preparation of trypsin is permanently inactivated when it is heated above 70°. Solutions of crystalline trypsin behave quite differently; they may be heated to boiling in acid solution, provided such boiling is brief, and recover their activity when fully cooled. The work of Northrop indicates very definitely that the inactivation of the enzyme by acid, heat, alkali, etc., is due to the denaturation of the enzyme protein. This is illustrated in Fig. 16. To different samples of a solution of pepsin at *p*H 5.4, alkali was added to bring them to various *p*H levels. The samples were then neutralized, the coagulated denatured protein was filtered off, and the activity and protein nitrogen content of the supernatant fluid in each sample were determined. The nitrogen content of the supernatant fluid represented, of course, the percentage of the pepsin protein that had not been denatured. It was found, as is evident from Fig. 16, that the percentage of undenatured or native protein was, in every case, exactly equal to the percentage of the enzyme that had not been inactivated.

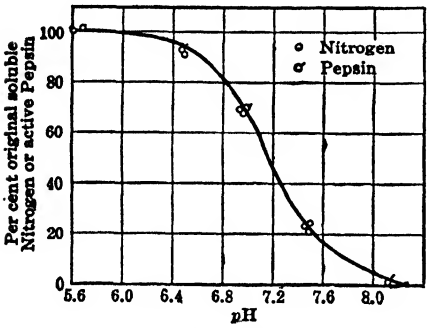


FIG. 16.—Percentage inactivation and percentage denaturation of pepsin at various *p*H and at 20° C. After J. H. Northrop, *J. Gen. Physiol.*, **13**, 739 (1929–30).

Solutions of crystalline pepsin inactivated in the manner described above may be partly reactivated when they are allowed to remain at *p*H 5.4 and 22° C. for 24 to 48 hours.

Crystalline trypsin is inactivated when it is heated in acid solutions (e.g., 0.1 *N*) to high temperature, 70° to 100° C., for brief periods; such inactivation is completely reversible when the trypsin solutions are cooled. Alkali also inactivates trypsin, but reversal here is more diffi-

[57] *J. Gen. Physiol.*, **14**, 713 (1931); **16**, 323 (1932); M. Kunitz and Northrop, *ibid.* **17**, 591 (1934); see also M. L. Anson and A. E. Mirsky, *ibid.*, **17**, 393 (1934).

cult; reactivation occurs when the sample is brought into dilute, salt-free acid, at *p*H 2.0, and allowed to remain for several hours.

Inactivation of enzyme may also occur during a reaction under conditions that are more normal than those described above. Thus, Northrop,[58] in a review of the data on the activity of catalase, has shown that the enzyme undergoes spontaneous inactivation during the reaction, that this inactivation follows a monomolecular course, and that, when it is taken into account, the course of the reaction may be mathematically formulated. Northrop has also pointed out that trypsin undergoes similar inactivation.

Another instance of enzyme inactivation during the course of the reaction occurs when dialyzed preparations of phosphatase are used to effect the hydrolysis of sodium-β-glycerophosphate. It has been pointed out that this inactivation is greater than the inactivation which the enzyme undergoes when it is kept under the same conditions (*p*H, presence of buffer, etc.) as exist in the hydrolysis, but without the presence of the substrate. The presence of α-amino acids and magnesium prevent such inactivation (O. Bodansky [24]).

Influence of Reaction Products. The decrease of the rate of reaction during the course of enzyme action is dependent not only on the factors so far discussed (decrease of substrate concentration, possible inactivation of enzyme), but also on the fact that the reaction products usually exert a retardant effect. This has been demonstrated for many enzymes. It may be illustrated by the data on invertase represented in Figs. 17 and 18. We note, in the former, the effect of varying concentrations of the different mutameric forms of glucose and fructose on the initial velocity of the hydrolysis of a 2 per cent sucrose solution. Thus a 4 per cent concentration of α-glucose retards the velocity 30 per cent; the same concentration of β-glucose retards the action 50 per cent. In Fig. 17 we note that the retardation by these sugars is less, the greater the initial concentration of sucrose. It may be appreciated that the precise formulation of the retardant effect during the course of the reaction is complicated and difficult. In a reaction in which, as in invertase hydrolysis, the liberated products undergo mutarotation, or other change, and thereby alter their retardant effect, an additional complication is introduced.

The acceleration of an enzymic reaction by its products has been reported in an unique instance: the hydrolysis of sucrose by honey invertase (Nelson and Cohn [59]). Both glucose and fructose accelerate, but the effect of the former is more marked. The acceleration by glucose is maximal at a concentration of 0.6 per cent and amounts to an increase of about 60 per cent in the initial velocity of the hydrolyzing sucrose.

The retardant effect of the reaction products of enzyme action is

[58] *Ibid.*, **6**, 417, 429, 439 (1923).

[59] J. M. Nelson and R. S. Anderson, *J. Biol. Chem.*, **69**, 443 (1926); Nelson and D. J. Cohn, *ibid.*, **61**, 193 (1924); Nelson and C. T. Sottery, *ibid.*, **62**, 139 (1924).

explained by Michaelis [45] on the basis that such reaction products, being structurally similar to the substrate, are also capable of combining with the enzyme. This combination results in removing active enzyme from the sphere of action.

Reaction Products and the Reversibility of Enzyme Reactions. In the usual chemical reaction, the products interact to reform the original reactants. There is, in short, a reverse reaction. When we say

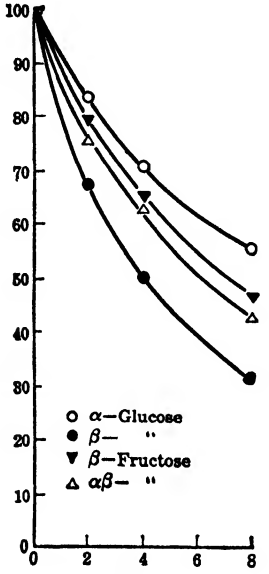


FIG. 17.

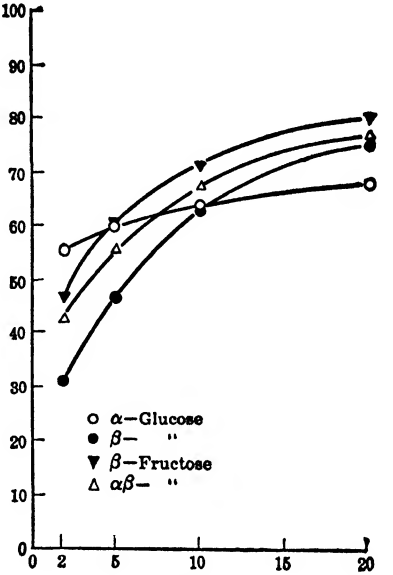


FIG. 18.

FIG. 17.—Effect of varying glucose and fructose concentrations on initial velocity of hydrolysis of 2 per cent sucrose by yeast invertase. Abscissa, hexose concentration per cent. Ordinates, velocities of hydrolysis of sucrose. Data of Nelson and Anderson. Curves reproduced from J. B. S. Haldane's "Enzymes," 1930, p. 47, through the courtesy of author and publisher, Longmans, Green and Company.

FIG. 18.—Effect of varying sucrose concentration on initial rate of its hydrolysis by yeast invertase in presence of 8 per cent glucose or fructose. Abscissa, sucrose concentration per cent. Ordinates, velocities of sucrose hydrolysis as percentages of velocity in absence of glucose. Data of Nelson and Anderson. Curves reproduced from J. B. S. Haldane's "Enzymes," 1930, p. 47, through the courtesy of author and publisher, Longmans, Green and Company.

that a reaction has been completed, we mean that, after a certain time, it has come to equilibrium: the velocity with which the original substances are reacting to form the products is exactly equal to the velocity of the reverse reaction.

The reversibility of many enzyme reactions has been demonstrated. Thus, Croft Hill [60] in 1898 treated a very concentrated solution of glu-

[60] *J. Chem. Soc., London*, **73**, 634 (1898).

cose with yeast maltase and obtained a disaccharide. Danilewski,[61] Robertson,[62] Wasteneys and Borsook,[63] among others, have reported the synthesis of proteins from products of peptic proteolysis. Josephson [64] studied in detail the synthesis of methyl-β-glucoside from glucose and methyl alcohol in the presence of emulsin. Synthesis of an ester in the presence of esterase was reported by Kastle and Loevenhart,[65] and of fat from glycerol and fatty acids by Hamsik.[66]

In most direct reactions taking place in the presence of enzymes, a very large fraction, usually more than 99 per cent of the original reactants are changed. If the reaction is pictured as

$$A + B \rightleftarrows C + D,$$

then the equilibrium constant,

$$K = \frac{[A][B]}{[C][D]},$$

is of a very low order of magnitude. This means that in the reverse reaction, in order to obtain any appreciable concentrations of A and B, we must start with high concentrations of C and D, and allow the reaction to proceed for a considerable time.

Synthetic processes in the organism are, of course, fully as important a link in its functioning as the processes through which the large molecules of ingested material, or the molecules brought to the tissues, are broken down. As we have seen, the study of enzymes has contributed a good deal to the elucidation of these latter processes. In comparison, the study of the rôle of enzymes in synthetic processes has been quite limited.

Perhaps the closest approach to such study has been made by Wasteneys and Borsook.[63] These investigators found that synthesis from concentrated solutions of products of proteolysis was not noticeable without the presence of enzyme. The creation of an emulsion by the use of certain substances, such as benzaldehyde, toluene, chloroform, etc., led to slight, but definite, synthesis. The combination of pepsin, an emulsifying agent, and high concentration of protein digests was most favorable to definite synthesis.

We may, of course, imagine, that the important conditions for synthesis which the above experiments indicate may exist within the cell in certain regions and at certain times. The effect of the emulsions indicates the importance of adsorption, or surface phenomena. In connection with this, Wasteneys and Borsook [63] have advanced further speculations regarding the synthesis from proteolytic digests: "Varia-

[61] Cited by Wasteneys and Borsook, *Colloid Symposium Monograph*, **6**, 155 (1928).
[62] *J. Biol. Chem.*, **3**, 95 (1907); **5**, 493 (1908–09).
[63] *Physiol. Rev.*, **10**, 110 (1930).
[64] *Z. physiol. Chem.*, **147**, 155 (1925).
[65] *Am. Chem. J.*, **24**, 491 (1900).
[66] *Z. physiol. Chem.*, **59**, 1 (1909); **65**, 232 (1910).

tion in physical and chemical properties with the emulsifying agent employed suggests a possible mechanism by which the many proteins of the organism may be synthesized, as they are, *in vivo*, from a common substrate."

Wasteneys and Borsook named the synthetic product which they obtained "plastein." They found it to be digested at *p*H 1.7 by pepsin with the liberation of carboxyl and amino groups and were, in general, inclined to regard it as a protein. Folley [67] has studied the nature of this synthetic product further. He submitted a sample of a preparation to Svedberg for determination of its molecular weight, and this investigator reported that the maximum molecular weight was 1000, and probably that the actual weight was not more than a few hundred. On the basis of this and other data, Folley is inclined to believe that plastein cannot be considered a protein, but rather a polymerized peptone.

Coenzymes, Activators, Accelerants, Inhibitors, Poisons, etc. Numerous observations have been made on the way in which certain substances present in enzyme preparations, or added to them, affect the enzyme activity. Such substances have been classified, of course, on the basis of the type of effect which they produce. Investigation during the past few years, however, has given more insight into the mechanism of these actions and should, therefore, alter somewhat the basis of classification.

Coenzymes. The term coenzyme has been applied to heat-stable, crystalloidal organic substances specifically associated with an enzyme. In 1906, Harden and Young [68] showed that zymase, which mediated the fermentation of glucose, could be separated into two fractions. One of these was colloidal and thermolabile (*apozymase*); the other (*cozymase*) was thermostable, dialyzable, and organic in nature. Euler and his coworkers [69] continued the study of this reaction. They found that washed and dried yeast was itself incapable of fermentation, but was active when mixed with the cozymase in the form of cooked yeast juice. They suspected the nucleotide nature of the cozymase and finally identified its basic constituent with nicotinic acid amide. Schlenk and Euler [69] have proposed the following constitution for cozymase.

Nicotinic acid amide—ribose—O—(P : O)—O—(P : O)—O—ribose—adenine

It is thus a diphosphopyridine nucleotide.

Warburg and associates [70] investigated the system which was involved in the oxidation of hexosemonophosphoric acid to phosphohexonic acid. They purified the coenzyme which was part of the system and finally found it to be composed of one molecule of adenine, one of β-nicotinic

[67] *Biochem. J.*, **26**, 99 (1932).

[68] *Proc. Roy. Soc. (London), B*, **77**, 405 (1906).

[69] *Z. physiol. Chem.*, **177**, 237 (1928); **184**, 163 (1929); **238**, 233 (1936); **240**, 113 (1936); **241**, 239 (1936); **242**, 215 (1936); *Naturwissenschaften*, **24**, 794 (1936).

[70] *Biochem. Z.*, **282**, 157 (1935); **285**, 156 (1936).

amide, three of phosphoric acid, and two of pentose. It is thus very similar to the cozymase involved in fermentation, but is instead a triphosphopyridine nucleotide.

We shall, in a later chapter, note the rôle of adenylic acid and adenylpyrophosphate as coenzymes in the reactions proceeding in muscle tissue.

The term "coenzyme" has acquired a more definite meaning as a result of the work of Warburg and associates. Thus the mechanism which Warburg and his co-workers have pictured for the oxidation of hexosemonophosphate involves (*a*) the transfer of two atoms of hydrogen from the substrate to the phosphopyridinenucleotide (which has been called coenzyme), (*b*) the transfer of two atoms of hydrogen from the reduced coenzyme to the yellow enzyme (p. 135), (*c*) the transfer of two atoms of hydrogen from the reduced yellow enzyme to oxygen with the formation of hydrogen peroxide. We see that in steps (*a*) and (*b*) a pyridine ring, and in steps (*b*) and (*c*) an isoalloxazine group, is involved in the transference of hydrogen. The isoalloxazine group is considered as part of the prosthetic group of an enzyme. It would therefore seem more consistent also to consider, as Warburg has done, the phosphopyridine nucleotide as a prosthetic group combined with a protein moiety, the *zwischenferment*, just as the lactoflavinphosphoric acid, is combined with its protein. The union of the phosphopyridine nucleotide is, however, more easily dissociated.

Kinases. It has been an observation of long standing that pancreatic juice, collected without contact with the intestine, does not digest protein. However, the addition of an extract of intestinal mucosa endows the pancreatic juice with tryptic activity. The intestinal substance which converts trypsinogen, the inactive or proenzyme form of trypsin, into the active form was called *enterokinase*. The mechanism of the conversion has been studied at great length, but, as will be seen, such studies produced confusing results, and more precise knowledge waited upon the crystallization of the various enzymes.

Kunitz and Northrop [71] found that, when a crystallized preparation of trypsinogen was dissolved in neutral solution, it formed some trypsin immediately. This trace of trypsin then catalyzed the change further so that the reaction proceeded autocatalytically and very rapidly. Amorphous preparations of trypsinogen contained a substance which inhibited the action of trypsin. This inhibition in turn prevented the conversion of trypsinogen into the active form. Activation was possible in these cases only by the addition of *kinase*, or by large amounts of trypsin.

The conversion of chymotrypsinogen to chymotrypsin (this enzyme like trypsin attacks protein in a weakly alkaline solution) has also been studied by Kunitz and Northrop.[71] They found that the rate was proportional to both the trypsin and the chymotrypsinogen concentrations when crystallized preparations were used. Pepsinogen, the

[71] *J. Gen. Physiol.*, **19**, 991 (1936); J. H. Northrop, *Physiol. Rev.*, **17**, 144 (1937).

precursor of pepsin, has no proteolytic activity. In slightly acid solution it becomes converted into pepsin; the reaction is autocatalytic and hence is caused by pepsin itself. Anson [72] has studied the conversion of the precursor of carboxypeptidase to the active enzyme.

Activators. There are many substances which increase the activity of enzyme preparations to which they are added. Some of these actions have been studied in detail and indicate that, at least in these cases, the phenomenon of "activation" may be more properly termed "reactivation" or "prevention of inactivation."

Thus we have seen (p. 133) that the activation by hydrogen sulfide or hydrocyanic acid of crystalline urease, which had previously been inactivated by aeration, heavy metals, etc., could be looked upon as a regeneration of the sulfhydryl group of the enzyme. Acceleration of papain-proteinase action by hydrocyanic acid may similarly be looked upon as neutralization of the inhibiting effect of a heavy metal present as an impurity. The activity of phosphatase is increased by the presence of magnesium ion; about 0.01 to 0.001 *M* produces an optimal effect, and it is only in the presence of such concentration of magnesium that the enzyme activity is proportional to the enzyme concentration. Manganous, cobaltous, nickelous, and ferrous ions increase the activity of arginase when it has previously been subjected to oxidation or to hydrogen sulfide, and, in this connection, Hellerman [26] has pointed out that the activity of the arginase may be connected with the property of the metallic ion to coordinate with suitable molecules, possibly the enzyme and the substrate.

α-Amino acids, in low concentrations, have been found to increase the activity of urease, amylase, and phosphatase. This effect has been studied in detail for phosphatase and has been found to be a prevention of the inactivation of the enzyme during its action. This finding justifies the view of Nord [73] that many activators are preventors of inactivation.

Other examples of activation, however, cannot be explained in the above manner, and in still others the mechanism has not yet been sufficiently studied. Phosphate ion accelerates the processes of fermentation probably because it takes part in the reactions. Arsenate ion seems to activate the enzymes in this process. The presence of salts increases the activity of amylases of animal origin. Thus pancreatic amylase is inactive in phosphate or sulfate solutions, but is optimally activated by 0.02 *M* sodium chloride, 0.10 *M* nitrate ion, or 0.15 *M* thiocyanate ion.

Retardants, Inhibitors, Poisons. We have already noted the retardant effect of reaction products, or of substances similar in chemical structure to the substrate. Such substances have been studied with regard to the extent of their retardation at different concentrations of

[72] *Ibid.*, **20**, 663, 777, 781 (1937).

[73] *Proc. Am. Soc. Biol. Chem.*, **7**, lviii (1927); *J. Biol. Chem.*, **74**, lviii (1927).

substrate—that is, as to whether they compete with the substrate for combination with the enzyme, or whether they merely inactivate a portion of the enzyme.

The inactivating effect of metals has already been noted. The salts of mercury, silver, and gold are most effective. Those of copper, zinc, lead and cadmium are less so, and still less effective are the salts of aluminum, chromium, manganese, cobalt, and nickel. The factor of concentration is, of course, very important. Magnesium, for instance, accelerates in low concentrations, retards in high. Some of these metals have been called "poisons" with regard to enzyme action. Yet we know that their effect, marked as it may be, is, in many cases, completely reversible.

Enzymes are in general rendered less active by protein precipitants such as picric and phosphotungstic acids, by aldehydes, amines, alkaloids, and by fluoride. It is, however, important to recognize that these effects are dependent on the concentration of the reagent, vary in degree for various enzymes, and, in some cases, are reversible. These effects have been explained on a chemical basis. For instance, the inhibiting action of certain amines corresponds to their affinity for aldehydes; the inhibiting action of aldehydes is assumed to be due to a combination with an amino group in the enzyme. Confirmation of such explanations awaits further detailed work with crystalline preparations of enzymes.

There are many other substances which inhibit enzyme activity. A most important group are the oxidizing agents such as iodine, hydrogen peroxide, oxygen in the presence of cupric ion, potassium permanganate, etc. It has been shown, for papain and urease, that the controlled use of oxidizing agents leads to completely inactive preparations which are presumably the oxidized forms of the enzymes, and that such inactivation is completely reversible by the use of reducing agents (cysteine, reduced glutathione, etc.).

Effect of Light and Other Radiations. This factor has received considerable attention. With regard to the visible spectrum, Green[74] reported activation of salivary amylase by red and blue light. Activation of this type appears to depend in many instances on the presence of oxygen. This has been found to hold for saccharase, amylase, peroxidase, and catalase. Bernheim and Dixon[75] studied the reduction of methylene blue by hypoxanthine in the presence of xanthine oxidase and found that activation was due to the production of hydrogen peroxide.

Ultraviolet light usually causes inactivation of enzymes, but occasionally activation has been reported. The inactivation effect, on crystalline pepsin, has recently been studied more precisely. Northrop[76] noted that the decrease in activity was proportional to the decrease

[74] *Proc. Roy. Soc.* (*London*), **61**, 25 (1897).
[75] *Biochem. J.*, **22**, 113 (1928).
[76] *J. Gen. Physiol.*, **17**, 359 (1933-34).

in the protein nitrogen. No denatured protein, however, appeared, and the effect of the ultraviolet paralleled the breakdown of the protein. Northrop employed these results as another evidence of the association of the enzymic properties with the crystalline protein. Gates [77] investigated the absorption spectrum of this enzyme, finding that the total absorption in the ultraviolet region paralleled the degree of inactivation and was especially marked between 2400 and 2750 Å. The rate of inactivation is sensitive to *p*H and is greater at lower *p*H levels.

The effect of radium emanations has also been studied, particularly by Hussey and Thompson.[78] β- and γ-rays produce marked inactivation. Northrop noted that, in crystallized pepsin exposed to radium bromide, the inactivation was proportional to the decrease of protein N, as with ultraviolet light.

Reports on other radiations do not indicate decisive effects. It is stated that X-rays produce effects on some enzymes and no alterations in others. Recently radio waves have been studied,[79] but under most of the circumstances investigated no definite effect on enzyme activity can be attributed.

Autolysis. When some water containing an antiseptic like chloroform or toluene is added to plant or animal tissue, disintegration and, sometimes, liquefaction of the tissues results. This phenomenon, noted by Salkowski [80] in 1889, reveals the activity of enzymes in the tissues which can act on the constituents of those tissues and is known therefore as *autolysis*. It may often be noted *post mortem* in various tissues. This action of the tissue enzymes within the tissue is also evident in the body during life, either physiologically (atrophy in the mammary gland after lactation), or pathologically (for example, acute yellow atrophy of the liver).

When a tissue is subjected to autolysis, the respiratory enzymes are soon inactivated while the hydrolytic enzymes attacking carbohydrates and proteins continue undiminished in their activity and may even be activated. As autolysis proceeds, protein, the major constituent of the tissue, disappears; the tissue grows progressively more acid, lower split products of protein increase in concentration, and among the end-products are found ammonia, amino acids, and phosphate.

The proteolytic enzyme present in the tissue has been named *cathepsin* and its properties studied in some detail. It, like preparations of papain, is activated by hydrogen sulfide, hydrocyanic acid, and compounds containing the sulfhydryl group (Hellerman [22]).

The autolytic capacities of various tissues and the conditions governing autolysis have been studied in detail by several investigators. Glandular tissues, such as kidney, intestinal mucosa, pancreas, and

[77] *Ibid.*, **18**, 265, 279 (1934).
[78] *Ibid.*, **5**, 647 (1922–23); **6**, 1, 7 (1923–24).
[79] A. P. Forjaz, *Biochem. Z.*, **283**, 53 (1935).
[80] *Z. physiol. Chem.*, **13**, 506 (1889).

liver, autolyze more rapidly than connective tissues such as muscle or bone.[81] Since products of autolytic proteolysis, including amino acids, are known to affect the activity of several enzymes, it is important to evaluate the effect of such products in judging the activity of enzyme extracts prepared by an autolytic procedure.

Study of Enzyme Action in Tissues. Upon reviewing our discussion of enzymes, we may state that the trend of the studies we have considered has been to ascertain the properties of the pure enzymes, to evaluate the effect upon their activity of physiologically important substances, and, by such methods, ultimately to determine their action within the body. However, in recent years, a more direct attack has been made upon this last phase.

Warburg[82] introduced the method of studying the respiratory activity of tissue slices taken from the living organism, or immediately after death. His co-workers and other investigators have studied in detail the respiratory activity of such material in a variety of physiological and pathological conditions. Krebs[83] has recently applied the method of tissue slices to the study of the action of deaminases in various organs. We shall discuss some of his work in other connections in a later chapter.

Another approach to the problem of the rôle of enzymes *in vivo* has been the attempted correlation of enzymatic activity with the histological structure. In 1931 Linderstrøm-Lang and Holter[84] introduced a series of ultramicrochemical methods whereby they were able to determine accurately substances present in a concentration of a few micrograms. Cylinders of frozen tissue, of the order of 2 mm. in diameter, were obtained by means of a cork borer. From these cylinders, slices 10 to 25μ in thickness were obtained with microtomes. Alternate sections were used for histological study and the determination of enzyme activity. In this way, "enzymatic histochemical profiles" of various organs such as the stomach, duodenum, and kidney have been obtained, and enzymic activity has been correlated with histological structure. This technique has been adopted by various schools of investigators and is being used very actively.

[81] H. C. Bradley, *Physiol. Rev.*, **2**, 415 (1922); H. G. Wells, "Chemical Pathology," Philadelphia, 1925.

[82] O. Warburg, collected papers in "Über die katalytischen Wirkungen der lebendigen Substanz," Berlin, 1928.

[83] *Ann. Rev. Biochem.*, **5**, 247 (1936).

[84] *Ergeb. Enzymforsch.*, **3**, 309 (1934).

CHAPTER VII

DIGESTION

Of the substances taken as food, the fats, proteins, and carbohydrates are ingested in forms not readily absorbed into the circulation and hence are not available to the organism for purposes of nutrition until they have become converted into small particles which can diffuse through the intestinal wall. The carbohydrates are changed into monosaccharides, the fats into glycerol and fatty acids, and the proteins into amino acids. In a measure, hydrolytic changes occur during the process of cooking, but the major part of the disintegration of the foodstuffs takes place in various portions of the alimentary tract. The chemical transformations by which foods are converted into small diffusible particles constitute the process of digestion. The enzymes which take part in this process are secreted by certain glands and pass into the different parts of the digestive tract, the mouth, stomach, and small intestine. In the following paragraphs the fate of the foodstuffs in digestion will be traced.

Salivary Digestion. The saliva is a mixed secretion produced mainly by three pairs of glands, the submaxillary, sublingual, and parotid, and to a lesser degree by the mucous membrane and the buccal glands of the mouth, throat, and esophagus. It is a viscous, frothy, slightly opalescent fluid, containing many substances. The salivary glands possess two kinds of cells: the serous or albuminous, which secrete a fluid containing protein and enzyme; and the mucous cells, which secrete a ropy fluid containing the glycoprotein mucin. A mixed secretion is obtained from the submaxillary gland which has both serous and mucous cells. The sublingual glands are chiefly mucous; and the parotid, chiefly serous.

Salivary flow is normally caused by a variety of stimuli. Psychic secretion is brought about by a reflex stimulation. The excitation, which may be caused by the sight, smell, or thought of food or by the hearing of sounds associated with the preparation of food, travels along afferent paths, the stimuli being transmitted along efferent pathways to the salivary glands. During vomiting, the abdominal fibers of the vagus nerve are stimulated and cause an increased flow as a result of reflex stimulation of the salivary centers. Mechanically, salivary flow may be induced by the presence in the mouth of solid particles, such as food, sand, or paraffin. Dry food calls forth a greater amount of saliva than moist food. Many chemical agents, such as acids, salts, and flavored substances, stimulate the secretion of saliva.

The total amount of saliva secreted in twenty-four hours by a normal man has been calculated to be about 1500 cc. Among the many factors that influence the daily volume of saliva are (*a*) the amount of water consumed, (*b*) the amount of food intake and the degree of its mastication, and (*c*) the character of the food. Chewing and smoking usually increase the flow of saliva.

Considering the complex nervous mechanism and other factors dominating the secretory and excretory functions of the salivary glands, it is not surprising that the composition of saliva should vary appreciably in different individuals, and in the same individual from time to time, and at different times during the day. On the basis of analyses of various investigators, the water content of human mixed saliva may be set at approximately 99.42 per cent; the amount of total solids is 0.58 per cent. Of the latter, about one-third is composed of inorganic constituents and the remainder of organic substances, including mucin, enzymes, and epithelial cell débris. The information regarding the inorganic composition of human saliva is relatively scanty. Such data as are available indicate that diet does not alter it markedly. The ingestion of chlorides does not affect the chloride concentration in the saliva significantly. On the other hand, the administration of sufficient inorganic phosphate to produce an increased concentration in the blood increases its excretion by the salivary glands.[1]

Starr [2] analyzed 610 specimens of human saliva, obtained from 228 healthy, normal subjects, and found the reaction to vary from *p*H 5.75 to 7.05. In 86 per cent of the analyses, the variations were within a narrower range, namely, *p*H 6.35–6.85. That the reaction of the saliva is usually slightly acid has also been reported by Henderson and Millet,[3] who observed, moreover, that the salivary *p*H falls just before meals and remains low just after meals. Between meals, the reaction of the saliva approaches neutrality. The belief that an acid reaction of the saliva is harmful and that it is desirable to change it from acid to alkaline is probably without scientific basis. In fact, the saliva is a well-buffered mixture and it is practically impossible to change its

[1] The inorganic composition of dog's saliva has been studied somewhat more comprehensively. E. J. de Beer and D. W. Wilson (*J. Biol. Chem.*, **95**, 671 [1932]) have compared the inorganic composition of the blood serum and of the parotid saliva of dogs under a variety of conditions. The following are data obtained in a control experiment, the dog being under amytal anesthesia and secretion being stimulated by pilocarpine. The values (except for *p*H) are in milliequivalents per liter; the results for saliva represent the averages of the right and left parotid secretions.

	Na	K	Ca	Cl	HCO_3	*p*H
Serum	130 6	4.2	5.2	110.3	26.4	...
Saliva	128.9	11.4	9.8	101.3	60.3	7.7

[2] *J. Biol. Chem.*, **54**, 55 (1922).

[3] *Ibid.*, **75**, 559 (1927).

reaction for periods longer than a few minutes by the addition of even moderate amounts of either acid or base.[4]

Mechanically, owing to the water and mucin, the saliva aids in the mastication of foods and serves as a solvent for some of the constituents. Chemically, the saliva takes part in the digestion of carbohydrates. The enzyme *ptyalin* in the saliva is capable of hydrolyzing starch into a variety of dextrins and ultimately into maltose which may be further digested to glucose by maltase, a second enzyme found in the saliva in small amounts.[5]

The conversion of starch into simpler products by the saliva may be demonstrated *in vitro*. This process may be followed by means of the well-known starch-iodine test. As the starch is hydrolyzed, on testing with iodine, the original blue color exhibited by starch gives way to a reddish color due to the so-called erythrodextrins. As the process continues, the digest yields paler tints, and finally it fails to yield any color whatever with iodine. Concurrently, the content of reducing sugars increases. The methods employed in studying starch digestion may be found in laboratory manuals of physiological chemistry.

In brief, salivary digestion consists in the transformation, with the aid of ptyalin, of a portion of the starch of the diet into simpler polysaccharides and maltose. As food remains in the mouth for a relatively short period, very little digestion occurs even of such carbohydrate-rich foods as bread or mashed potatoes. Carbohydrate digestion by the salivary enzymes may continue for some time in the stomach or until the food comes in contact with the hydrochloric acid of the gastric juice which inactivates the ptyalin. There is present in the parotid secretion a proteinase, resembling trypsin. Its rôle in digestion, if any, is negligible. There are no fat-splitting enzymes in the saliva.

Gastric Digestion. Gastric digestion is concerned primarily with the partial disintegration of the protein of the diet. This is accomplished by the enzyme pepsin in the presence of hydrochloric acid. A second important digestive function is the clotting of milk by rennin.

Among the more significant of the earlier contributions to our knowledge of gastric digestion are those of Réaumur (1752) and of Spallanzani (1783). These investigators studied gastric secretion in birds, fishes, and mammals, and demonstrated that gastric juice is acid, that it prevents putrefaction, that the juice has digestive properties *in vitro*, and that the process of digestion is essentially a chemical one.

[4] A. L. Bloomfield and J. G. Huck, *Bull. Johns Hopkins Hosp.*, **31**, 118 (1920); V. R. Carlson and McKinstry, *Dental Cosmos*, **66**, 840 (1927); G. W. Clark and K. L. Carter, *J. Biol. Chem.*, **73**, 391 (1927); Editorial, *J. Am. Med. Assoc.*, **92**, 899 (1929).

[5] Ptyalin, as well as other amylases (pancreas, malt), acting on starch does not produce a 100 per cent conversion into maltose. The yield is usually about 75 per cent, owing to the formation of an intermediate product, presumably a dextrin, which is resistant to the action of amylase.

In 1825, William Beaumont,[6] a young American surgeon, began a classical investigation of digestion, which lasted until 1833, on a patient with clinical gastrostomy. The patient's name was Alexis St. Martin, and he was first observed by Beaumont in 1822. Little was known at that time concerning the mechanism of gastric secretion. Beaumont was a very careful worker and painstakingly studied the factors that influence the flow of gastric juice. He found that the presence of food in the stomach stimulates gastric secretion, and that irritating condiments, alcohol, anger, fear, and fever diminish it. He failed to observe, however, that gastric secretion may occur in the absence of food. Beaumont recorded observations showing that gastric flow may be induced by mechanical stimulation of the gastric mucosa, a view which, though later disputed by Pavlov, has been confirmed in more recent investigations.[7]

Some time before Beaumont began his experiments, Prout, and later Tiedemann and Gmelin, reported that the acid in the stomach was hydrochloric acid. Beaumont, however, surmised that the gastric juice contained active chemical agents other than hydrochloric acid. He thus anticipated by several years the actual discovery of pepsin by Schwann and by Wasmann.

Following the work of Beaumont, experimental methods were introduced for the study of gastric secretion. The removal of juice by means of a stomach tube is a method still employed clinically. Juice may also be collected readily from an artificial fistula. A gastric fistula is made by cutting an opening into the stomach and sewing the cut portions to the abdominal wall. The collection of pure gastric juice, uncontaminated by food, was first made possible by Heidenhain. His method consisted in cutting through the walls of the stomach, sewing the flaps into a pouch which was then sewed to the abdominal wound. By this operation, however, most of the extrinsic and intrinsic nerve connections were severed. Hence, there remained the possibility that the gastric secretion formed in the pouch was not normal. This difficulty was overcome by Pavlov, who devised an improved technique [8] for making an isolated gastric pouch.

[6] "Experiments and Observations on the Gastric Juice," Plattsburg, 1833.

[7] Ivy, Lim, McCarthy, and Farrell, *Am. J. Physiol.*, **72**, 203, 232 (1925).

[8] The following is Pavlov's description of the operation (Pavlov, "The Work of the Digestive Glands," translated by Thompson, London, 1902 edition, p. 11): "The first incision, which begins in the fundus of the stomach, 2 cm. from its junction with the pyloric end, is carried in the longitudinal direction for 10 to 12 cm., and divides both the anterior and posterior walls. A triangular flap is thus formed, the apex of which lies in the long axis of the stomach. A second incision is made exactly at the base of this flap, but only through the mucous membrane, the muscular and peritoneal coats remaining intact. The margins of the mucous membrane all around these incisions are separated for a little way from the submucous tissue: on the side of the stomach for a width of 1 to 1½ cm.; on the side of the flap for 2 to 2½ cm. The raised edges of mucous membrane belonging to the large stomach are applied to each other for half their width and sewn together. Out of the piece which belongs

The following diagrams illustrate the operation:

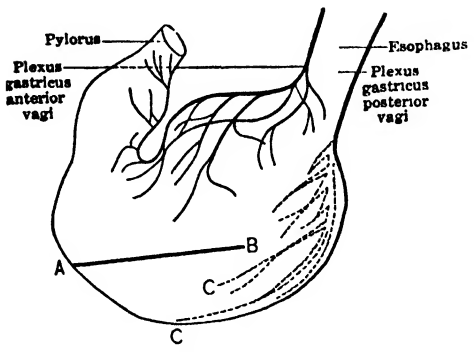


FIG. 19*a*

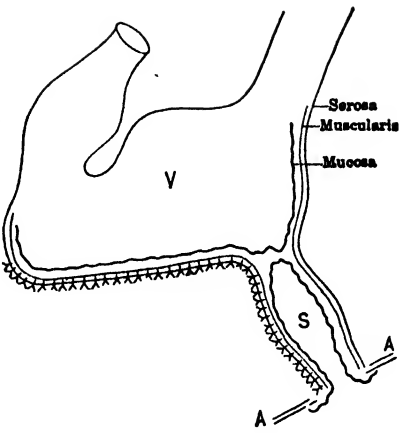


FIG. 19*b*

FIG. 19*a*.—*A–B*, line of incision; *C*, flap for forming stomach pouch of Pavlov.

FIG. 19*b*.—*V*, cavity of the stomach; *S*, Pavlov's pouch. *S* is separated from *V* by a double layer of mucous membrane. *A*, abdominal wall. (After Pavlov.)

By means of the Pavlov pouch, it is possible to obtain gastric juice which is similar in character to that secreted in the main stomach and which is not contaminated by saliva or food material. In his numerous experiments, Pavlov employed dogs with gastric pouches and dogs with gastric and esophageal fistulas. If the esophagus is divided and the two ends sutured to the skin, an opening is formed. Food swallowed by dogs with esophageal fistulas does not reach the stomach but falls through the upper end of the fistula; hence, this is known as sham feeding.

The essential results of Pavlov's work on gastric digestion may be summarized as follows:

1. The amount of juice secreted is normally proportional to the amount of food and depends to a considerable extent on the character of the food to be digested.

2. In sham feeding there is no chemical or mechanical stimulation. The stimulation to gastric flow is psychic.[9] Psychic secretion of gastric juice may be caused by the sight or smell of food or by established conditioned reflexes. If, at the time of feeding, a bell is rung, a conditioned

to the flap a cupola is formed. Both the stomach and the margins of the flap are then closed by sutures along the edges of the first incision. A septum is thus made between their respective cavities, consisting of two layers of mucous membrane: one, that of the cupola, being intact, the other stitched along the middle."

Modifications of this technique have been introduced by various workers. See, for example, the improvements suggested by F. Hollander and G. R. Cowgill, *J. Biol. Chem.*, **91**, 151 (1931); E. E. Jemerin, *Proc. Soc. Exp. Biol. Med.*, **38**, 139 (1938).

[9] Psychic stimulation of gastric secretion in man was studied by A. T. Carlson, "The Control of Hunger in Health and Disease," Chicago University Press, 1916.

reflex is established in the course of time, so that eventually the mere ringing of the bell, without the presentation of food, causes gastric secretion.

3. Mechanical stimulation, according to Pavlov, produces no secretion. This is not in accord with Beaumont or with the recent work of Ivy.

4. Many substances, especially those having a flavor, produce secretion.[10]

The Origin of Hydrochloric Acid. The *parietal* or *border* cells found in the gastric glands are associated with the secretion of hydrochloric acid. In the concentration in which it is formed, the acid may be expected to be injurious to the cell protoplasm, and it therefore seems reasonable to infer either that acid is not actually formed within the parietal cells or that it diffuses quickly, as it is formed, into the intracellular canaliculi and thence into the lumina of the glands. The opinion that the production of hydrochloric acid does not occur intracellularly is based partly on the interpretation usually given to the experiments of Harvey and Bensley.[11] These investigators injected potassium ferrocyanide, as well as certain indicator dyes (cyanamin bichloride, neutral red), into animals and later examined microscopically the gastric mucosa, which had taken up these substances. The presence of free acid was demonstrable on the internal surface of the stomach and in the fovea of the glands, but the canaliculi of the parietal cells, as well as the cytoplasm, remained alkaline in reaction. These results were essentially confirmed by Dawson and Ivy,[12] who, however, pointed out that cyanamin gives an alkaline reaction above *p*H 3. Under their experimental conditions, the cytoplasm and canaliculi were alkaline to cyanamin, but acid to neutral red, from which it was concluded that the reaction of the active parietal cell is slightly acid (*p*H 3 to *p*H 6.8). This conclusion is in harmony with other experimental evidence (FitzGerald,[13] Hammett,[14]

[10] In their contribution to the subject, Lim, Ivy, and McCarthy (*Quart. J. Exptl. Physiol.*, **15**, 13 [1925]) have analyzed the factors concerned in the excitation of gastric secretion as follows: (1) The cephalic phase, heretofore referred to as the "psychic secretion" demonstrated by Pavlov, which is excited chiefly by the taste, smell, and mastication of palatable food, and by sight, thought, or hypnotic suggestion of palatable food. The term "psychic secretion" is rejected because it is not necessarily psychic, having been shown to occur in the absence of the cerebral cortex. (2) The gastric phase, in which mechanical and chemical stimuli are effective. (3) The intestinal phase, in which the stimuli are certain chemical substances acting in the intestine. Ivy, Lim, and McCarthy (*Quart. J. Exptl. Physiol.*, **15**, 55 [1925]) have demonstrated that the intestinal phase of gastric secretion is due to the action of the products (e.g., peptone, amino acids, and amines) of digested complex food substances and, apparently, not to the food in its raw state (meat, carbohydrates, and neutral fat).

[11] *Biol. Bull.*, **23**, 225 (1912).

[12] *Am. J. Physiol.*, **76**, 158 (1926).

[13] *Proc. Roy. Soc.* (*London*), *B*, **83**, 56 (1910).

[14] *Anat. Rec.*, **9**, 21 (1915).

Collip [15]). There is obvious need for more precise data on the subject, but even though existing knowledge is limited, it nevertheless seems to justify the assertion that the cytoplasm of the parietal cell, and the contents of the canaliculi, are only slightly acid even during activity and that the strong acidity characteristic of the gastric juice is first observed at the mouth of the tubules of the secreting glands.[16] Concerning this there is more or less general agreement, but the question of how the hydrochloric acid is formed is much more unsettled.

The problem becomes somewhat clearer if it is realized that the composition of the gastric secretion is related to that of the blood. The chloride concentration of the former is 160 to 165 milliequivalents (0.16 *N*–0.165 *N*), which is essentially the concentration of the total base in the blood, suggesting that the two fluids are at least in osmotic equilibrium with each other. Since other sources of the H^+ and Cl^- ions may be excluded, it becomes apparent that they must have their origin in the blood.

One possibility is that the hydrochloric acid arises from an interchange of ions between monobasic sodium phosphate and sodium chloride, as indicated by the equation:

$$(1) \qquad Na^+H_2PO_4^- + Na^+Cl = Na_2^{++}HPO_4^{--} + H^+Cl^-$$

Although there is insufficient evidence to establish this mechanism, certain experimental data are very suggestive. When a solution of phosphates is enclosed within a parchment membrane, there results a diffusion of acid with a consequent increase in the alkalinity of the fluid within the dialyzer due to the more rapid diffusion velocity of acids than of alkaline salts. A similar mechanism has been suggested for the formation of hydrochloric acid by the gastric mucosa from the alkaline phosphates of the blood. An objection to this view has been raised by Robertson,[17] who states that this theory proves too much, for, by parity of reasoning, all the secretions of the tissues should be acid in reaction, whereas, actually, the majority of the secretions are alkaline.

A more plausible theory is that the interchange of ions occurs between carbonic acid and sodium chloride, as represented by equation (2).

$$(2) \qquad H^+HCO_3^- + Na^+Cl^- = Na^+HCO_3^- + H^+Cl^-$$

[15] *Univ. Toronto Studies, Physiol. Series*, No. 35.

[16] It is of interest to note that Claude Bernard (1813–78) injected animals with a solution of iron ammonium citrate and at the same time with potassium ferrocyanide. Owing to the presence of free acid, the gastric contents were deeply stained with Prussian blue (ferric ferrocyanide). The staining extended to the foveola, but the lumen of the tubules and the cells lining them were quite free from the blue stain. Cited by H. C. Bradley, *Yale J. Biol. Med.*, **4**, 399 (1932).

[17] T. B. Robertson, "Principles of Biochemistry," Lea & Febiger, Philadelphia, p. 366, 1924.

This view is supported by a number of observations. Dodds and McIntosh[18] found that during gastric secretion the CO_2 content of the blood increases and that there is a concomitant rise of the alveolar CO_2 tension. Both diminish as gastric secretion subsides and the production of pancreatic juice, which is alkaline in reaction, becomes predominant. Because of the sudden demand for chloride at the commencement of digestion, its concentration in the blood is temporarily lowered.[19] In addition to the removal of chloride by the gastric glands there is a marked shift into the corpuscles, owing to the rise of the blood CO_2 tension (see p. 277). The reduction in plasma chloride may therefore become pronounced. However, the loss is soon recovered, partly by the release of chloride from the tissue spaces and partly through reabsorption from the digestive tract.

The interdependence of hydrochloric acid secretion and the CO_2 content of the blood has been demonstrated by Apperly and Crabtree,[20] who were able to vary the concentration of acid in the gastric juice by changing the concentration of the bicarbonate in the blood. Indeed, gastric secretion may be inhibited if the blood CO_2 is diminished sufficiently (Browne and Vineberg [21]), as in acidosis, or by hyperventilation.

Secretion of large amounts of hydrochloric acid in the stomach is accompanied by an increase of base in the blood. This often occurs to such an extent that, despite the loss of alkali in the pancreatic and intestinal secretions, sufficient base remains to give the urine an alkaline reaction. The change in the reaction of the urine, following food intake, from the normal acid reaction to one that is alkaline, is referred to as the *alkaline tide.*

Even more direct evidence of liberation of base in the gastric mucosa, simultaneously with the secretion of hydrochloric acid, has been presented by Hanke.[22] In experiments on dogs in which gastric secretion was stimulated by the injection of histamine, the gastric venous blood contained more base, bound as bicarbonate and with protein, than was present in the arterial blood. The difference was in proportion to the rate of secretion and in fact was approximately equivalent to the amount of acid formed.

An experiment which is considered to have some bearing on the problem of acid formation is that of T. B. Osborne.[23] He dissolved edestin in sodium chloride solution and later precipitated it with a stream of carbon dioxide. The precipitate contained edestin in combination

[18] *J. Physiol.*, **57**, 139 (1923).

[19] E. C. Dodds and E. S. Smith, *ibid.*, **58**, 157 (1923); L. Martin, *Ann. Internal Med.*, **6**, 91 (1932).

[20] *J. Physiol.*, **73**, 331 (1931).

[21] *Ibid.*, **75**, 345 (1932).

[22] M. E. Hanke, R. E. Johannesen, and Maude E. Hanke, *Proc. Soc. Exptl. Biol. Med.*, **28**, 698 (1931).

[23] *Am. J. Physiol.*, **5**, 180 (1901).

with hydrochloric acid, whereas the solution contained $NaHCO_3$. Obviously, in the presence of the protein edestin the reaction represented by the equation $NaCl + HHCO_3 = NaHCO_3 + HCl$ was facilitated.

Behavior similar to that exhibited by edestin may be demonstrated with red blood corpuscles. If these are washed with isotonic solution of sodium chloride until the washings are neutral, then suspended in neutral sodium chloride solution and treated with a stream of carbon dioxide, it is found that the solution becomes alkaline and the corpuscles richer in chloride. From these observations, it has been inferred that the secretion of an acid juice depends upon the existence in the secreting cells of a protein capable of decomposing sodium chloride in the presence of carbon dioxide, the appearance of the free hydrochloric acid in the secretion being attributable to the indiffusible character of the positively charged protein ion. The validity of this suggestion remains to be determined by further study.

If it is assumed that, in the acid-forming cells, the hydrochloric acid is combined with protein or some other cell constituent to which the cell membrane is impermeable, an explanation for the secretion of free hydrochloric acid may then be based on Donnan's theory of membrane equilibria. The original state may be represented by the following diagram:

$$\begin{array}{c|c} R^+ & H^+ \\ Cl^- & OH^- \end{array}$$

R represents the positively charged protein ion (or other cell constituent). The membrane being permeable to the Cl^-, H^+, and OH^- ions, an interchange of ions will occur, so that at equilibrium the situation will be as follows:

$$\begin{array}{c|c} R^+ & H^+ \\ Cl^- & Cl^- \\ OH^- & OH^- \end{array}$$

This theory, according to Donnan,[24] receives support from the observation that under certain conditions hydrochloric acid may actually be "secreted" across a membrane. Donnan is of the opinion that "the proper ampholyte can easily give rise by this mechanism alone to a concentration of hydrogen ions in the external liquid as great as that found in the gastric juice." This view is not shared by most physiologists.

The formation of acid in animal organisms is not limited to the gastric mucosa. The salivary glands of the mollusc *Dolium galea* pro-

[24] *J. Chem. Soc.*, **99**, 1554 (1911); **105**, 1941 (1914); **115**, 1313 (1919); cited by Gortner, "Outlines of Biochemistry," p. 285, 1929.

duce a secretion containing 4–5 per cent sulfuric acid.[25] Certain related species of molluscs produce aspartic acid in large concentration.

The Acidity of the Gastric Juice and Its Regulation. The constancy of the hydrochloric acid concentration of the gastric juice as it is *secreted* has been the subject of much discussion. Proponents of the so-called Heidenhain-Pavlov theory have adopted the view that a relatively constant acidity is characteristic of the gastric secretion. The opposing view is based on Rosemann's [26] studies and his explanation that the presence of cations in gastric juice is due to the incomplete conversion of neutral chlorides to hydrochloric acid by the parietal cells, from which follows the conclusion that even normally the acidity is subject to conspicuous variations. For example, it is implied that the concentration of acid decreases with the falling off of the secretion (i.e., in its later stages).

The evidence which has accumulated in recent years is overwhelmingly in favor of the Heidenhain-Pavlov hypothesis. Experimental as well as clinical studies have shown that the secretion of the parietal cells is probably an isotonic solution of hydrochloric acid. According to Hollander,[27] the ideally pure parietal secretion contains no neutral chloride, inorganic phosphate, or significant amounts of any other substance. He has estimated that the concentration of acid is approximately 0.17 *N* (170 millimolar, or about 0.62 per cent). The *p*H is approximately 0.88.

Gastric contents as collected contain a variable amount of neutral chloride, bicarbonate, phosphate, etc. As these constituents occur also in juice obtained from an isolated gastric pouch, it stands to reason that some factor, other than regurgitation of alkaline duodenal contents, must be responsible for the neutralization of part of the acid. From the data at hand, it is probable that the gastric mucosa itself, but not the parietal cells, secretes a non-acid fluid, containing neutralizing agents. Mixing of the two solutions results in gastric juice as we know it. In such a mixture, only the chloride would be expected to remain relatively constant; the concentrations of hydrochloric acid and of base would

[25] Many years ago (1868) Horsford (cited by H. C. Bradley, *Yale J. Biol. Med*, **4**, 399 [1932]) observed that, when $CaHPO_4$ was subjected to dialysis, the dialyzate grew acid, whereas the contents of the dialyzing bag became alkaline and $Ca_3(PO_4)_2$ precipitated. An even earlier experiment of this type is that of Graham, who dialyzed potassium bisulfate against water. The dialyzate became acid owing to the diffusion of sulfuric acid. In terms of our present knowledge the reaction may be represented as follows:

$$\left.\begin{array}{r} K^+HSO_4^- \\ \longrightarrow \\ HO^-H^+ \end{array}\right| \rightarrow H_2SO_4$$

$$(KOH + KHSO_4 = K_2SO_4 + HOH)$$

[26] *Arch. ges. Physiol. Pharmacol.*, **118**, 467 (1907).

[27] *J. Biol. Chem.*, **104**, 33 (1934); *Am. J. Digestive Diseases Nutrition*, **3**, 651 (1936–37).

vary, depending on the relative proportions of the two secretions. These relations are illustrated diagrammatically in Fig. 20. Comparison of these diagrams with the composition of the blood serum (p. 273) shows that the secretions of the gastric mucosa and the blood have essentially the same total ionic content and are therefore in osmotic equilibrium with one another. The mixed gastric juice has a somewhat lower electrolyte content, owing to the release of CO_2 in the reaction of the acid with bicarbonate. However, it is to be noted that the chloride concentration of the mixed gastric juice remains at approximately

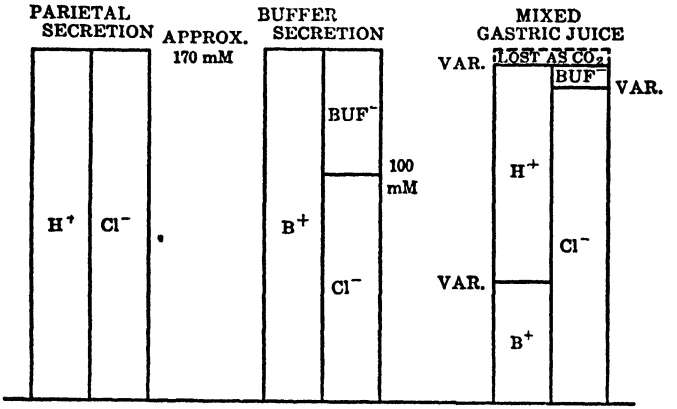


FIG. 20.—Diagrams illustrating the acid-base composition of parietal secretion and pure gastric juice formed by mixing the two secretions in variable proportions. After F. Hollander, *Am. J. Digestive Diseases and Nutrition*, **3**, 651 (1936–37).

0.165 *N* (165 millimolar) and that it is subject to very little variation, being much more constant, therefore, than the hydrochloric acid concentration which may vary from practically zero to 0.16 *N* (160 milliequivalents).[28, 29]

[28] The values obtained for the cat, dog, and man show good agreement. The problem of the constancy of gastric acidity has been especially well presented by the following authors: Hollander and Cowgill, *J. Biol. Chem.*, **91**, 151, 481 (1931); Hollander, *Am. J. Physiol.*, **98**, 551 (1931); *J. Biol. Chem.*, **104**, 33 (1934); *Am. J. Digestive Diseases Nutrition*, **3**, 651 (1936–37); J. L. Gamble and M. A. McIver, *J. Exptl. Med.*, **48**, 837 (1928); L. Martin, *Ann. Internal Med.*, **6**, 91 (1932), *Bull. Johns Hopkins Hosp.*, **55**, 57 (1934).

[29] The chloride-ion concentration of the gastric juice cannot exceed 165 ± 5 m. eq. unless the electrolyte content of the blood is raised. It has been demonstrated by Gilman and Cowgill (*Am. J. Physiol.*, **99**, 172 [1931–32]; **103**, 143 [1933]; **104**, 476 [1933]) that in dogs, as a result of water deprivation and the consequent dehydration, there was a definite rise in the electrolyte content of the blood. Accompanying the variations thus produced by alternate dehydration and hydration of the animal, parallel changes in the chloride content of the secretion were observed. In one experiment the gastric chloride rose to 183.2 m. eq. The maximum acidity did not, however, greatly exceed 160 m. eq. In short, these results indicate that the osmotic pressure of the blood is the limiting factor determining the chloride concentration and the minimum *p*H of the gastric secretion.

As has been mentioned, the concentration of hydrochloric acid, as it is formed, is comparatively high—about 0.6 per cent. It does not remain long at this level, but is rapidly diminished to a concentration which normally varies between 0.15 and 0.25 per cent. In part the neutralization is accomplished by the so-called buffer secretion of the gastric mucosa (Hollander). In all probability the mucus secreted by the gastric mucosa is of considerable importance. Its effect in reducing acidity has been studied by McCann,[30] who found that in the resting stomach the acid may be formed at a rate sufficiently slow that all the acid produced combines with the mucus. Bolton and Goodhart [31] in a recent discussion of the mucus factor in the automatic regulation of the acidity of the gastric contents state that " the only means possessed by the normal stomach, whereby it is able to reduce the acidity of its contents, is by the secretion of mucus." The saliva has not only a definite neutralizing, but a diluting effect as well. Foodstuffs, particularly the proteins, combine with hydrochloric acid. Finally, regurgitation of alkaline fluid from the intestine may be a factor at-times in the neutralization of gastric acidity.

Stimulation of Gastric Secretion; the Gastric Hormone. In the fasting stomach there occurs a continuous secretion of gastric juice; the rate is probably relatively low and subject to variation, though on this point there is admittedly not much information. Certain it is, however, that secretion is rapidly increased by a variety of stimuli, those associated with the taking of food being of particular interest.

The secretion due to psychic factors (the cephalic phase of gastric secretion described by Ivy) is usually referred to as " appetite juice." It is a discontinuous secretion and obviously significant in initiating gastric digestion. About half an hour after food-taking the rate of secretion is greatly augmented. It is not due to reflex action since it may be evoked after cutting both vagi. Nor is it due to mechanical stimulation, the presence of water or of undigested food materials. Broths, meat extract, meat juices, and the products of protein digestion, including certain amino acids, stimulate secretion. Products of starch hydrolysis do not exert an appreciable effect, and fats seem to inhibit secretion. Ivy and Javois [32] have studied the effect of protein-split products, including amino acids and certain amines, and found some of these to be potent gastric secretagogues. A certain degree of specificity was observed. For example, β-alanine was a powerful excitant, whereas α-alanine acted but feebly.

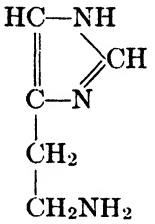
These substances contribute to the chemical stimulation of gastric secretion. But there is apparently a more important factor. Many workers have shown that the injection of extracts prepared from the pyloric mucous membrane causes increased secretion of gastric juice.

[30] *Am. J. Physiol.*, **89**, 483 (1929).
[31] *J. Physiol.*, **77**, 287 (1933).
[32] *Am. J. Physiol.*, **68**, 182 (1924).

The effect has been attributed to a specific secretagogue, called *gastric secretin*, or *gastrin*, described by Edkins.[33]

Gastrin is also present in the mucosa of the duodenum, though in smaller amount, as shown by Keeton and Koch.[34] These investigators could not demonstrate its presence in other tissues. More recently Murray [35] studied the effect of extracts prepared from various regions of the duodenum (cat). According to her report, only the sections which contained Brunner's glands yielded potent preparations. Attempts have been made to isolate "gastrin." Koch, Luckhardt, and Keeton [36] prepared moderately active preparations from the pyloric mucosa and, though unable to isolate the active principle, nevertheless concluded from its chemical properties that it might be an imidazole compound related to, if not identical with, histamine.

According to reports from Ivy's laboratory,[37] gastrin is probably histamine. From acid extracts of the pyloric mucosa Ivy and co-workers have isolated this substance as the picrate. The possible formation of histamine in the process of isolation is emphatically ruled out, and evidence is presented to show that it is the sole secretory excitant. *Histaminase* (an enzyme which destroys histamine) was found to destroy the effectiveness of the crude "gastrin" solutions.



Histamine

The subcutaneous injection of histamine is followed by a copious gastric secretion which is very acid in character. This remarkable property was discovered by Popielski [38] in 1920 and has since been investigated in both experimental and clinical studies of gastric function. A relatively small amount of histamine is required, from 0.5 to 1 mg. (or 0.1 mg. per 10 kg. of body weight) being generally employed to produce the desired response. In man the maximum secretion of acid is usually attained between 30 and 45 minutes after the injection

[33] *Proc. Roy. Soc.* (*London*), *B*, **76**, 376 (1905); *J. Physiol.*, **34**, 133 (1906).
[34] *Am. J. Physiol.*, **37**, 481 (1915).
[35] *J. Physiol.*, **69**, 48 (1930).
[36] *Am. J. Physiol.*, **62**, 508 (1920).
[37] *Ibid.*, **101**, 331 (1932); see also M. S. Kim and A. C. Ivy, *ibid.*, **105**, 220 (1933); A. C. Ivy, "Gastrointestinal Principles," *J. Am. Med. Assoc.*, **105**, 506 (1935).
[38] *Arch. ges. Physiol.*, **178**, 214 (1920).

of histamine. In the dog, according to Gilman and Cowgill,[39] the maximum rate of secretion is reached in 30 to 45 minutes. The acid and total chloride concentrations reach their maximum values at about the same time.

Histamine apparently stimulates only the parietal, or acid-secreting, cells. Pilocarpine, on the other hand, promotes the secretion of the organic substances and enzymes. This effect is best elicited when the administration of this drug is preceded by an injection of histamine because in that way the organic substances are " washed out " from the lumen of the glands and the furrows of the mucosa. Vineberg and Babkin[40] state that the combination of the two excitants produces a synthetic gastric juice approaching normal.

Inhibition of Gastric Secretion; Enterogastrone. The presence of undigested fat in the intestine exerts an inhibiting effect on gastric secretion and motility. As this effect may also be demonstrated in a transplanted gastric pouch, it seems reasonable to attribute it to a hormone mechanism. Accordingly, a number of physiologists have adopted the view that the presence of fat in the intestine causes the liberation from the mucosa of a hormone, *enterogastrone*, which is specifically concerned in the motor and secretory inhibition of the stomach.[41] It has been suggested (Quigley) that a similar mechanism may be involved in the inhibition of gastric motility when glucose is present in the upper part of the intestine.

The Enzymes of the Gastric Juice. Three enzymes participate in gastric digestion: *pepsin*, *rennin*, and *lipase*. The origin of lipase is somewhat disputed, one view being that it is secreted with the other enzymes in the stomach, while according to another view it is derived from regurgitated pancreatic and intestinal juice. The hydrogen-ion concentration in the stomach is usually unfavorable both for the emulsification of fat and for the action of lipase, so that the amount of gastric fat digestion is ordinarily slight, being limited to the partial hydrolysis of the highly emulsified fats and the more soluble glycerides of the lower fatty acids, such as are present in egg yolk, butter, and milk.

In the absence of acid in the stomach, digestion may be largely intestinal in type, being due to the pancreatic and intestinal enzymes contained in the regurgitated intestinal contents.

The *chief cells* (*Hauptzellen*) have been definitely associated with the secretion of pepsin and rennin. The acid furnishes a favorable hydrogen-ion concentration for the action of pepsin. Formerly it was considered that this enzyme was present in the cells in an inactive or *zymogen* form, called *pepsinogen*, which was presumably converted into the active form

[39] *Am. J. Physiol.*, **97**, 124 (1931).

[40] *Ibid.*, **97**, 69 (1931); *Am. J. Digestive Diseases Nutrition*, **1**, 715 (1935).

[41] T. Kosaka and R. K. S. Lim, *Chinese J. Physiol.*, **7**, 5 (1933); A. C. Ivy, *J. Am. Med. Assoc.*, **105**, 506 (1935); J. P. Quigley and K. R. Phelps, *Am. J. Physiol.*, **109**, 133 (1934).

by hydrochloric acid.[42] The evidence for this was that neutral extracts of gastric mucosa, which had not been previously treated with acid, were more resistant to the action of alkali than activated pepsin, the latter being quickly decomposed in an alkaline medium. According to Waldschmidt-Leitz the differences in the behavior of pepsin and its zymogen are attributable to the effects of hydrogen-ion concentration. Of perhaps greater significance are the observations of Northrop (p. 150) that crystalline pepsin may be completely inactivated by making the solution alkaline to *p*H 10.5. If the solution is then acidified to *p*H 5.4 and allowed to stand at 22° C. for 24 to 48 hours, the enzyme solution recovers some of its activity. These results indicate the reversibility of inactivation of pepsin and suggest that the difference between the active and inactive forms may reside in slight physical or chemical modifications of the protein which is pepsin. This need not be interpreted, however, as a final settlement of the problem.

Pepsin digests protein to its primary cleavage products, the proteoses and peptones. The food does not remain in the stomach sufficiently long for further transformation. But even in prolonged *in vitro* digestion by pepsin the amount of the simpler polypeptides and free amino acids liberated is small. This has led to the supposition that pepsin is restricted in its action to some specific linkage or linkages in the protein molecule. However, peptic digestion is associated with the liberation of free carboxyl and amino groups, which are said to be liberated in approximately equivalent amounts. This would indicate that pepsin involves the O=C—NH group, but in view of the limited amount of such cleavage it has been assumed that only certain specific bindings are thus affected. Obviously the mode of action of pepsin has not been settled and requires further study.[43]

Peptic digestion may be followed by observing the change in the amount of coagulable protein, or the increase in incoagulable nitrogen. The progress of digestion may also be measured by Sørensen's titration

[42] Because of its alleged effect on "pepsinogen," hydrochloric acid was formerly classified as an *activator*. An activator has been conventionally defined as a substance which converts the inactive form of an enzyme into its active form. Other examples will be encountered later. In contradistinction, an agent which accelerates the action of an already active enzyme has been defined as a *co-ferment*, or *coenzyme*. Haldane ("Enzymes," Longmans, Green & Co., London, 1930, Chapter VII), however, employs the term "activator" for non-specific substances which permit or *increase* the activity of an enzyme, thus including most of the agents formerly classified as coenzymes. He states further that the activation may be primarily physical, as in the case of a variety of colloids which activate lipases, or chemical, as in the case of anions which activate animal amylases.

Pepsinogen from swine's gastric mucosa has been isolated in crystalline form and converted into pepsin by acidification. The pepsin produced in this way on crystallization proved to be identical with the crystalline enzyme prepared from commercial pepsin (R. M. Herriott and J. H. Northrop, *Science*, **83**, 469 [1936]).

[43] In this connection the reader is referred to the summary by Max Bergmann, "Proteins and Proteolytic Enzymes," *Harvey Lectures*, Series **31**, 37 (1935–36).

(increase in COOH groups) or the Van Slyke amino nitrogen determinations. Another method commonly employed especially with certain substrates consists in determining the changes in viscosity of the digests. As digestion progresses, the viscosity decreases.

Rennin. Rennin is secreted by the gastric mucosa and is said to be especially abundant in young animals. The essential feature of the process of milk clotting, with which rennin is concerned, is the hydrolysis of the casein molecule. Calcium is essential for rennitic action, and if it is removed by treating the milk with oxalate, clotting does not occur on the addition of rennin. When to milk so treated calcium is added, clotting occurs. If milk, to which rennin has been added after the removal of the calcium, is allowed to stand for some time and then boiled to destroy the enzyme, and calcium is finally added, clotting occurs. This shows that the rennin must have acted on the casein in some way in the absence of calcium. Many theories have been advanced for the clotting process of milk, but tentatively the changes may be represented by the following equations:

$$\text{Casein} + \text{rennin} = \text{paracasein (soluble)}$$

$$\text{Paracasein} + \text{Ca} = \text{Ca-paracasein (insoluble)}$$

The action of rennin may also be represented by the equation:

$$\text{Ca-casein} + \text{rennin} = \text{Ca-paracasein}$$

The curdling of milk may be brought about likewise by the addition of acid. In the precipitation of calcium caseinate by hydrochloric acid, isoelectric casein, which is insoluble, is formed:

$$\text{Ca-casein} + \text{HCl} = \text{casein} + \text{CaCl}_2$$

The Question of the Identity of Pepsin and Rennin. Hammarsten considered rennin to be a special proteolytic enzyme distinct from pepsin. This view was supported by the fact that pepsin preparations without rennitic action and rennin preparations without peptic activity have been obtained. A contrary view was proposed by Pavlov. It postulated that both activites were due to the same enzyme. This conception was based partly on the wide distribution and coexistence of pepsin and rennin in plants and animals. Indeed, the property of clotting milk has been attributed not only to pepsin, but also to trypsin and the autolytic proteases of tissues.

Obviously the non-identity of pepsin and rennin can be established only by the complete separation of the two enzymes. The crystalline pepsin of Northrop (p. 130) possesses rennitic action. However, Tauber and Kleiner [44] obtained an active preparation of rennin from the

[44] *J. Biol. Chem.*, **96**, 745 (1932); **106**, 501 (1934); compare with R. Ege and E. Lundsteen, *Biochem. Z.*, **268**, 164 (1934).

fourth stomach of the calf. Their product is relatively pure, is practically devoid of peptic activity, and is much more powerful in coagulating milk than any rennin previously described. It differs from pepsin in being not a protein, but a thioproteose, in containing neither calcium nor phosphorus, in being soluble in 0.04 per cent hydrochloric acid, non-coagulable by heat, and diffusible through a dialyzing membrane. Unlike pepsin it yields negative Millon and Hopkins-Cole reactions and weaker xanthoproteic and biuret tests. The isoelectric point of rennin is given as *p*H 5.4, whereas that of pepsin is *p*H 2.75. Rennin is more resistant than pepsin to destruction by acid. Propepsin (pepsinogen) and prorennin differ in respect to the *p*H at which they become activated, according to Tauber and Kleiner. This has been contradicted by other workers.

Summarizing the available information, it may be stated that pepsin, as represented by Northrop's crystalline preparation, has both proteolytic and rennitic action, but that a separate and distinct enzyme is elaborated by the gastric mucosa which possesses only rennitic activity.

Gastric Motility, Hunger Contractions. Carlson [45] has shown that hunger is a sensation accompanying movements of the stomach, and that in conditions where hunger is abnormally keen, the contractions of the stomach are increased both in height and frequency. It has been suggested that the increased gastric tonus and contractions are associated with the reduction of the tissue glycogen. A relationship has even been assumed to exist between the blood-sugar level and gastric motility, but this has been denied recently by Quigley and Halloran,[46] who were unable to modify spontaneous gastric motility in dogs by the intravenous injection of glucose.

Gastric hunger contractions may be augmented by fasting, moderate exercise, hemorrhage, and exposure to cold. Insulin stimulates motility of the stomach, as well as of other parts of the gastrointestinal tract. Marked reduction of gastric motility, and in fact long-continued gastric atony, accompanied by loss of appetite (anorexia), occur in dogs (and probably in man) deprived of the antineuritic factor (vitamin B_1). This has been the subject of a long series of investigations by Cowgill and associates and will be considered in another connection.

Discharge of Food from the Stomach. Factors which increase the intensity and frequency of the contractions of the stomach, such as hunger, fasting, or the administration of thyroid substance or insulin, also hasten the rate at which the food is discharged into the intestine. Constriction waves propel the semi-liquefied food, or chyme, toward the pyloric sphincter, which is apparently able to resist the passage of large or solid food fragments, but which opens from time to time to permit the passage of small amounts of chyme. On the basis of Cannon's work the

[45] A. J. Carlson, "The Control of Hunger in Health and Disease," University of Chicago Press, 1916.

[46] *Am. J. Physiol.*, **100**, 102 (1932).

stimulus for opening of the pyloric sphincter was until recently attributed to the accumulation of acid on the stomach side, whereas the stimulus for closure was thought to be the presence of acid on the duodenal side. During the period of relaxation, there probably is not only the ejection of material from the stomach, but the reverse passage of intestinal contents into the stomach. The view that the acid is the essential factor for the alternate opening and closing of the sphincter is no longer generally accepted, for it appears that contraction of the sphincter may be produced not only by acid, but also by mechanical irritation, and even by alkali. The stomach, moreover, can also empty itself of alkaline contents. Carlson and Litt [47] believe that ordinary sensory stimuli may induce contraction of the pylorus.

This view finds some support in the work of McCann.[48] In animals in which the pyloric sphincter is resected, there is only partial reduction in the efficiency of food retention in the stomach. This observation, adequately controlled by fluoroscopic examinations and fractional analyses of the gastric contents, has led McCann to conclude that the emptying of the stomach is not controlled by the sphincter alone, but that the whole pyloric antrum is actively engaged in the process. The vigorous tonic and peristaltic contractions of the pylorus observed early in digestion, McCann believes to be due to its irritability. As digestion progresses and the food is reduced to a semi-fluid consistency, this stimulus gradually diminishes, giving way to a progressive relaxation of the pars pylorica, including the sphincter, and results in the more rapid emptying of the stomach. Neither the free hydrochloric acid nor the products of digestion seem to be the specific influence for the relaxation.

The type and amount of food determine the rate of evacuation of the stomach. When fed separately, carbohydrate food remains in the stomach for a shorter period than protein food. Fat remains in the stomach for a longer period than proteins. The digestion of protein food is markedly delayed on a high-fat diet. In part, the differences in the rate of emptying of the stomach are due to the variations in the time required for mechanical disintegration and enzyme action.

Gastric Analysis. Departures from the normal composition of the gastric contents have long been considered of significance in clinical diagnosis. Accordingly, various methods and functional tests have been devised for the purpose of obtaining information on one or more of the following points: presence or absence of free hydrochloric acid and its amount; total acidity; peptic and rennitic activity; abnormal retention of food in the stomach, and the character, including the microscopic appearance, of the residuum; evidence of regurgitation, presence of blood, and organic acids, such as lactic and butyric, particularly the former.

[47] *Arch. Internal Med.*, **33**, 281 (1924).

[48] *Am. J. Physiol.*, **89**, 497 (1929); compare with B. A. McSwiney and L. N. Pyrah, *J. Physiol.*, **76**, 127 (1932).

Gastric flow may be stimulated by the ingestion of a so-called test meal. The one proposed by Ewald consists of a dry piece of toast or a roll and a cup of weak tea (about 250 cc.). The test is performed in the morning on an empty stomach; one hour after the meal, the complete stomach contents are removed (by means of a suitable stomach tube, such as the Rehfuss tube) and analyzed. The procedure may be modified to advantage by removing the stomach contents before giving the meal.

Dilute oatmeal gruel as the test meal (Boas) is also widely used. The Riegal test meal consists of beef broth, beefsteak, and mashed potatoes. A wide variety of other test meals have been proposed.

The information derived from a single analysis is, however, inadequate. To meet this objection, Rehfuss introduced a method of fractional analysis. The stomach is emptied and the contents kept for analysis; the meal is given, after which specimens are withdrawn at 15-minute intervals for one hour. Should it seem desirable to continue the test for a longer period (2 to 3 hours), the last specimens may be collected at intervals of 30 minutes.

Gastric secretion may also be stimulated by the administration of dilute alcohol, a procedure that has been applied clinically. Special advantages have been pointed out in the use of histamine (p. 172).

The methods of gastric analysis will be treated only briefly in this connection, as they belong more properly in manuals devoted to clinical laboratory diagnosis. A general inspection of the specimens withdrawn may yield useful information. For example, the stomach contents removed before the test meal may be abnormal in volume. Whereas ordinarily the amount of fluid in the resting stomach varies between 15 and 40 cc., it may be many times this as a result of obstruction. This would be further indicated by the presence of food particles. The test meal is normally evacuated in 1.5 to 2 hours. Should there be no evidence of the test meal in about an hour, it signifies increased motility. If, on the contrary, a residue is present after 2 to 3 hours, it shows diminished motility, or obstruction usually at the pyloric sphincter.

Although appropriate tests are available for the detection of blood and bile, these may be readily recognized grossly. A bright red color indicates recent bleeding, such as may occur from an ulcer or an esophageal varix. Bleeding due to these causes is profuse. Very frequently a small amount of blood may be detected. This results from the slight trauma that is often produced in passing the stomach tube. If bleeding occurs slowly, but continually, there may be present a considerable amount of blood possessing a color that has been likened to that of coffee-grounds. Considerable significance may also be attached to the odor. It may be characteristic of fermentation (lactic or butyric acid), or may be even more disagreeable, as in cancer, severe catarrhal gastritis, etc.

For analysis the stomach contents are usually strained through

cheesecloth. The free hydrochloric acid is determined by titration with standard alkali, using dimethylaminoazobenzene (Töpfer's reagent) as the indicator. This shows a change from red (acid) to salmon-pink and yellow at *p*H 3 to 4. The results are conventionally expressed in terms of the number of cubic centimeters of 0.1 *N* acid present in 100 cc. of gastric contents.

Not all the hydrochloric acid is free. Some occurs in combination with protein. By using phenolphthalein as the indicator, the value for total acidity may be obtained. Titration with alizarin as the indicator is supposed to represent the free hydrochloric acid plus the organic acids and acid salts. The difference between this titration and the one with Töpfer's reagent is a measure of the organic acidity. From the difference in the titration values with phenolphthalein and alizarin may be calculated the combined hydrochloric acid. For most purposes, the titrations for free and total acidity are sufficient.

Helmer and Fouts [49] have stressed that the value obtained by titrating to the phenol red end-point (*p*H 7.0) is more significant and gives a more precise measure of total acidity than titration to the phenolphthalein end-point, since the latter includes buffer substances other than acid.

Physiological and Pathological Variations. In considering physiological standards it is necessary to recall that the acidity of the gastric juice as it is secreted is close to and approaches as a maximum the value of 160 units or m.eq. (160 cc. 0.1 *N* acid per 100 cc.). This is reduced in various ways so that the total acidity ordinarily encountered after a test meal amounts to 60 to 90 units as a maximum, and depending on various factors previously discussed, the free acidity may be decreased to an average value of about 40 to 50 units.

Vanzant and associates [50] have analyzed the data of nearly four thousand test meals performed on individuals of all ages who were presumably free from gastrointestinal disease. The free acidity, which is low in childhood, increases rapidly up to the age of 20 years, when the adult values are reached. Achlorhydria (absence of free hydrochloric acid) was encountered in all age groups, but showed a steady increase in incidence up to the age 60 to 65 years. At the age of 60 years, 23 per cent of the men and 28 per cent of the women were achlorhydric. For other ages, too, achlorhydria was somewhat more frequent in women than in men. As an upper limit the free acidity rarely exceeded 90. This illustrates the broad range of values encountered in apparently normal individuals and emphasizes the necessity for caution and discrimination in the interpretation of the data of gastric analysis.

Excluding the cases of achlorhydria from the calculations Vanzant determined the mean value for free acidity for men between the ages

[49] *Am. J. Clin. Path., Technical Supplement*, **7**, 41 (1937). This paper summarizes the method of collecting gastric juice, as well as the various procedures used in its analysis (*p*H, pepsin, rennin).

[50] *Arch. Internal Med.*, **49**, 345 (1932).

of 20 and 40 years to be about 45 to 50 units. From this the level falls off to about 30 to 35 for aged men. The total acidity is ordinarily 15 to 20 units higher than the free acidity. In women throughout adult life the mean is approximately 35 units of free acidity, with an average of about 18 units for combined acid.

Fractional Analysis. About 40 per cent of normal individuals show the following response to a test meal: increase in free HCl, sometimes from an initial value which is as low as zero, to a maximum of 45 to 55 at the end of 60 to 90 minutes. This is followed by a rapid decrease in the next 30 to 60 minutes to a level of about 15 to 20 units. In about one-third of normal persons, a relative hypersecretion and hyperchlorhydria occur, with maximum values for free HCl of 90 to 100. On the other hand, a certain proportion (25 to 30 per cent) show a tendency to hypochlorhydria.

As an extreme of hypoacidity, there is the condition of achlorhydria or anacidity in which free hydrochloric acid is entirely absent. In true anacidity, even histamine fails to provoke any secretion and the total acidity rises very little, if at all. Occasionally a case is encountered showing an almost normal rise in total acidity, but the absence of free HCl. Neutralization by regurgitated duodenal fluid, or by mucus, secreted in abnormal amounts is the usual explanation. Pernicious anemia, gastric carcinoma, and chronic gastritis are among the conditions in which anacidity is a prominent feature.[51]

In hyperacidity, or hyperchlorhydria, the concentration of free acid may rise from an initial value of 20 to 40 to over 100 units, within 60 to 90 minutes, and remain at a relatively high level for several hours. Such a response is commonly obtained in gastric and duodenal ulcer.

The Histamine Test. The use of test meals in determining gastric function has certain limitations. Psychic elements, such as lack of appetite, distaste for the food, the speed of eating, and the buffer value of the meal are disturbing factors. Other factors are the amount of saliva swallowed, regurgitation from the duodenum, and the speed with which the stomach empties itself. Because of so many variables, repeated tests often yield divergent results. Another factor which prevents uniformity is that the test meal does not ordinarily provide a maximum stimulus. In discussing this problem Bloomfield and Polland,[52] as well as others, have emphasized the point that one criterion of a satisfactory functional test is that the stimulus which makes up the test must impose a load on the function to be tested. Only when there is a maximal stimulus, i.e., under strain, is it possible to demonstrate early or partial impairment in function.

The histamine test is of especial value in establishing true achlor-

[51] The reader is referred to A. L. Bloomfield and W. S. Polland, "Gastric Anacidity, Its Relations to Disease," The Macmillan Co., New York, 1933.

[52] *J. Am. Med. Assoc.*, **92** 1508 (1929); see also Klumpp and Bowie, *J. Clin. Investigation*, **12**, 1 (1933).

hydria. Its most serious limitation is that it gives no information concerning the motor activity of the stomach. Moreover, should it be firmly established that histamine is the gastric secretagogue, it will be possible to dismiss the objection raised that histamine is not a physiological stimulus.

A modification of the test, requiring two injections of histamine (double histamine test), has been proposed by Rivers, Osterberg, and Vanzant.[53] It is designed not only to determine the maximal potentiality of acid and pepsin secretion of gastric cells, but also to give information regarding the maintenance of increased secretory rates over longer periods of time.

Tests for Enzymes, etc. The absence of pepsin, as well as hydrochloric acid, is termed *achylia*. For the determination of peptic activity, a commonly used procedure is the Nierenstein and Schiff modification of Mett's method. Small glass tubes, filled with coagulated egg albumin, are introduced into small flasks containing a definite amount of gastric juice, adjusted to approximately 0.05 *N* HCl. Digestion occurs at both ends of the tube; the portion of the column digested at each end is measured and used as the basis for calculating the peptic activity.

Polland and Bloomfield [54] use an edestin solution as substrate. The undigested portion is precipitated in tubes designed for this purpose. In the method of Koch and McMeekin,[55] a suspension of egg albumin is incubated with a definite quantity of gastric juice for 2 hours. The change in the refractive index of the solution, or the increase in soluble nitrogen, is used as the basis of calculating the degree of peptic activity. This is expressed in milligrams of U. S. P. pepsin per cubic centimeter.

Rennin is rarely determined for purposes of clinical diagnosis. Qualitatively it may be readily detected by neutralizing the gastric juice and adding 5 drops of it to 5 cc. of fresh milk in a test-tube. If placed in an incubator or water bath at 40° C., clotting should occur within a few minutes. A simple quantitative procedure has been described by Helmer and Fouts.[49]

Evidence of regurgitation may be obtained by testing the gastric contents for bile, or the pancreatic enzymes, notably trypsin. The presence of blood is detected by appropriate tests described in laboratory manuals. Lactic acid occurs commonly in the later stages of gastric carcinoma, but may occur in non-malignant pyloric obstruction associated with achlorhydria. In the absence of hydrochloric acid, bacterial fermentation is unchecked.

Anti-enzymes. In view of the proteolytic action of pepsin, the question may be raised as to the failure of the stomach mucosa to digest itself. One explanation offered is that the gastric mucosa contains an

[53] *Am. J. Digestive Diseases Nutrition*, **3**, 12 (1936–7).
[54] *J. Clin. Investigation*, **7**, 45 (1929).
[55] *J. Am. Chem. Soc.*, **46**, 2066 (1924).

anti-enzyme which inhibits the action of pepsin. Another explanation is that the blood and lymph bathing the cells of the stomach have an alkaline reaction which is unfavorable to peptic digestion. It is obvious, however, that the latter explanation cannot hold in the case of the intestinal mucosa, which is not attacked by either the intestinal or the pancreatic enzymes despite the favorable reaction.[56] Many regard the mucins to be of much importance in preventing autodigestion of the gastric mucosa. Powdered mucin, prepared from hog's stomach, has been employed in the treatment of peptic ulcer.[57]

"Intrinsic Factor." In 1926, Minot and Murphy [58] announced that patients with pernicious anemia could be definitely benefited by feeding liver, or by the administration of liver extract. The increase in reticulocytes, which results within a few days after treatment is instituted, is followed by a progressive rise in hemoglobin and red cells until practically normal values are attained. The curative effect of liver in pernicious anemia and in certain related macrocytic anemias is sustained as long as treatment is continued.

This epoch-making discovery naturally led to the study of the possible underlying factors involved in the process of erythropoiesis. It was recognized by Castle [59] that the almost constant occurrence of achlorhydria in pernicious anemia was probably of significance in this connection, reflecting perhaps other deficiencies of the gastric mucosa. He demonstrated that a reticulocyte response, followed by an increase in red cells and clinical improvement, could be obtained by the administration of a digest prepared by incubating beef muscle with normal gastric juice at *p*H 5 to 7. This effect was not obtained by the administration of normal gastric juice alone, or of a pepsin digest of beef muscle, or of the product obtained by incubating beef muscle with the gastric juice from patients with pernicious anemia. These observations, supplemented by a considerable amount of other data, led Castle to conclude that the formation of the hemotopoietic principle depends on two factors, an " intrinsic factor " present in the gastric mucosa and an " extrinsic factor " found in certain foods, such as beef muscle.

When both factors are available, the anti-anemic substance is produced and stored principally in the liver. Except that it is concerned in the normal maturation of red cells, the manner in which it acts on the

[56] According to Banting and Gairns, *Am. J. Physiol.*, **94**, 241 (1930), normal serum contains an antitrypsin. The skin of white rabbits contains a substance which is specifically antagonistic to the oxidase found in the skin of black rabbits. Kirk and Sumner, *J. Biol. Chem.*, **94**, 21 (1931–32) immunized rabbits to crystalline urease and produced an antibody which definitely inhibited the hydrolysis of urea by urease *in vitro* as well as *in vivo*. See also S. F. Howell, "Antiurease Formation in the Hen," *Proc. Soc. Exptl. Biol. Med.*, **29**, 759 (1931–2).

[57] S. J. Fogelson, *J. Am. Med. Assoc.*, **96**, 673 (1931); *Arch. Internal Med.*, **55**, 7 (1935); M. S. Kim and A. C. Ivy, *J. Am. Med. Assoc.*, **97**, 1511 (1931).

[58] *J. Am. Med. Assoc.*, **87**, 470 (1926).

[59] W. B. Castle, *Harvey Lectures*, **30**, 37 (1934–35).

bone marrow has not been elucidated. It occurs in normal human livers and has been found in the livers of pernicious anemia patients treated with liver extract, but not in the livers of untreated patients.

The chemical nature of the hematopoietic principle has not been fully established. A recent report from Dakin's laboratory [60] indicates that it may be a peptide of molecular weight between 2000 and 5000. On hydrolysis it yields arginine, leucine, glycine, proline, hydroxyproline, and an acid resembling hydroxyglutamic acid. The constitution of Castle's extrinsic factor is at present unsettled, nor is there complete information concerning the intrinsic factor. Of considerable interest, however, is the report from the Thorndike Memorial Laboratory [61] that normal gastric juice incubated with casein at *p*H 7.4 (pepsin and trypsin excluded) increases the total soluble nitrogen. This activity was destroyed when the gastric juice was boiled for 5 minutes, heated to 70–80° C. for 30 minutes, or incubated at *p*H 1.5 for 72 hours at 40° C., procedures which destroyed at the same time the ability of the juice to form hematopoietic substance upon incubation with beef muscle. These properties suggest that the intrinsic factor may be a protein-splitting enzyme distinct from pepsin and trypsin. Recent studies by Lasch [62] support this view.

DIGESTION IN THE INTESTINE

Pancreatic and Intestinal Secretions

There is practically no absorption of food from the stomach. Accordingly, the partly digested material enters the small intestine where it is subjected to the further action of three separate secretions, the pancreatic juice, the intestinal juice or *succus entericus*, and the bile. The pancreas is a long, irregularly shaped gland lying close to the duodenum. In the adult, the organ usually weighs about 70–90 grams. Two secretions are formed by the pancreas, an internal secretion which is concerned with regulating carbohydrate metabolism, and an external secretion which has digestive properties and which is conveyed to the duodenum by one or more ducts.

Mechanism of Pancreatic Secretion. Pancreatic secretion, although, in part, under the control of the nervous system, does not seem to be influenced by psychic stimuli, as is the case with salivary and gastric secretion. It has been shown (Pavlov) that the presence of acid chyme in the intestine normally causes active secretion by the pancreas. This excitation Pavlov thought to be due to a reflex stimulation, since

[60] H. D. Dakin, C. C. Ungley, and R. West, *J. Biol. Chem.*, **115**, 771 (1936).

[61] F. H. L. Taylor, W. B. Castle, R. W. Heinle, and M. A. Adams, *Proc. Soc. Exptl. Biol. Med.*, **36**, 566 (1937).

[62] *Klin. Wochschr.*, **16**, 810 (1937); G. R. Minot and W. B. Castle, *Yearbook of General Medicine*, 1937, p. 346.

the pancreas is under the control of both the vagus and the splanchnic nerves. However, in 1902, Bayliss and Starling [63] were able to show that, even after nervous communication with the pancreas had been destroyed, secretion could be induced by the introduction of acid into the intestine. Working on the assumption that the secretory mechanism was under chemical control, Bayliss and Starling prepared an acid extract from the intestinal mucosa, and after neutralizing it, injected it into the circulation of dogs. This resulted in copious secretion of pancreatic juice.

This chemical mechanism is believed to consist in the transformation of a substance known as *prosecretin*,[64] present in the intestinal mucosa, into secretin. Secretin is a hormone, or " chemical messenger," which enters the circulation and is carried to the pancreas which it rouses to activity.

Of the many experiments which have confirmed the work of Bayliss and Starling, several reported by Ivy and Farrell [65] are especially convincing. These workers transplanted the tail of the pancreas of dogs, subcutaneously, beneath the mammary gland, and in the same animals made a Thiry fistula of the jejunum. When dilute acid was applied to the Thiry fistula, the pancreatic transplant was stimulated to secrete. As this occurred after ligation of the bile duct and injection of atropine (the latter inhibits gastric secretion), the secretion of the transplant could not have been due to bile or to gastric juice flowing into the duodenum. In another experiment, loops of the jejunum were transplanted subcutaneously in animals with a pancreatic transplant. The original blood and nerve supply to both transplants was then severed. Application of dilute acid to the intestinal transplant provoked copious secretion by the pancreatic transplant, whereas the application of water had no effect.

Bayliss and Starling prepared secretin by scraping the intestinal mucosa (duodenum and jejunum), grinding with sand, then boiling with 0.4 per cent HCl. The mixture was neutralized, acidified with acetic acid, cooled, and filtered. The filtrate contained the pancreatic secretagogue, but also many unknown substances.

Crude secretin preparations have a marked vasodilating effect as well as other physiological properties unrelated to pancreatic stimulation. The problem has therefore been to separate secretin, not only

[63] *J. Physiol.*, **28**, 325 (1902).

[64] The evidence for the pre-stage of secretin is inconclusive, although the observations of Still and Keith support this idea (cited by E. U. Still, *Physiol. Rev.*, **11**, 328 [1931]). Intestinal mucosa was frozen (the freezing destroys the cellular continuity of the tissue) and dropped into (*a*) hot 0.4 per cent HCl and (*b*) hot 0.9 per cent NaCl. The HCl extract contained twenty times as much activity as the saline extract. When the latter was acidified, its activity increased several times.

[65] *Am. J. Physiol.*, **77**, 474 (1926); **78**, 325 (1926); *J. Am. Med. Assoc.*, **89**, 1030 (1927); A. C. Ivy, J. I. Farrell, and H. C. Lueth, *Am. J. Physiol.*, **82**, 27 (1927).

from inert material, but also from other physiologically active substances. Luckhardt, Barlow, and Weaver [66] obtained an active preparation by the following simple method. An excised duodeno-jejunal loop was washed by passing through it a rapid stream of water. One end was then clamped and the loop filled with 0.4 per cent HCl and the other end clamped. After 30 minutes the acid was removed, neutralized, and filtered. The filtrate was an effective secretagogue and practically free from vasodilating substances. This extract has formed the starting-point for other investigators who have attempted to prepare more active products, with the ultimate aim of obtaining pure secretin.

Weaver, Luckhardt, and Koch [67] saturated the acid extract with sodium chloride. The flocculent precipitate which formed was dried and preserved. It was soluble in water, and was relatively free from vasodilatins. This salt concentrate has been subjected to further purification and study by Still [68] and Ivy.[69] Working independently and employing somewhat different methods these investigators obtained products of about equal potency. Their preparations are also pure, at least physiologically. Still has shown his product to be free from vasodilator substances, cholecystokinin (see p. 196), oxytocic and hypoglycemic substances. In addition to its effect on the pancreas, secretin seems to have a specific cholagogue action, i.e., it stimulates the hepatic cells to produce bile.

Mellanby [70] has likewise prepared an active secretin. His product has the percentage composition of a protein. Employing in part the same procedures as other investigators, Cunningham [71] has carried the process of purification still further and has recently reported the isolation in crystalline form of a secondary proteose which he believes to be the hormone secretin. Ågren [72] has also reported the crystallization of secretin, as the picrolonate. The hormone is basic in reaction (*p*H about 7.5), gives positive biuret and ninhydrin reactions, is digested by proteolytic enzymes, and is said to have a molecular weight of about 5000.

Composition of the Pancreatic Juice. The pancreatic juice is a clear liquid having an alkalinity corresponding to a *p*H of about 7.5 to 8.0.[73] The specific gravity is approximately 1 007 and subject to slight variation. Water constitutes about 98.7 per cent, the remainder

[66] *Am. J. Physiol.*, **76**, 182 (1926).

[67] *J. Am. Med. Assoc.*, **87**, 640 (1926).

[68] E. U. Still, *Am. J. Physiol.*, **91**, 405 (1930); *Physiol. Rev.*, **11**, 328 (1931); Still and J. La Barre, *Am. J. Physiol.*, **91**, 649 (1930).

[69] Ivy *et al.*, *Am. J. Physiol.*, **95**, 35 (1930).

[70] *J. Physiol.*, **66**, 1 (1928); *Proc. Roy. Soc.* (*London*), **111**, 429 (1932).

[71] *Biochem. J.*, **26**, 1081 (1932).

[72] *Skand. Arch. Physiol.*, **70**, 10 (1934).

[73] C. G. Johnston and E. G. Ball (*J. Biol. Chem.*, **86**, 643 [1930]) in a study of pancreatic juice obtained from permanent pancreatic fistulas in dogs observed a range of values between *p*H 7.16 and 8.04.

consisting of coagulable protein, organic substances, enzymes, and inorganic compounds.

Recent studies of the inorganic constituents are of particular interest. The concentration of base is fairly constant and is practically equivalent to the concentration in the blood plasma (about 160 millimoles per liter). Gamble and McIver [74] have reported the following data for the distribution of the various ions:

TABLE XXV

ACID-BASE COMPOSITION OF PANCREATIC JUICE FROM DOG

Base	Cc. 0.1 *N* per 100 cc.	*Acid*	Cc. 0.1 *N* per 100 cc.
$Na^{\cdot}$	148	Cl	81
$K^{\cdot}$	7	HPO_4''	1
$Ca^{\cdot\cdot}$	6	HCO_3'	79
	161		161

In these analyses the HCO_3' was taken to be the difference between the values for total base and for $Cl' + HPO_4''$. Mg, which was not determined, is present in relatively small concentration. Values between 0.3 and 0.7 mM. (millimoles) per liter were obtained by Johnston and Ball.[73]

Somewhat higher values for HCO_3' were obtained by Gamble and McIver in direct determinations after equilibration of the pancreatic juice with alveolar air.

Similar results were obtained in analyses of human pancreatic juice. The total base amounted to 164 mM. and the Cl content averaged 87 mM. per liter.

Pancreatic Digestion. Three proteolytic enzymes are present in the pancreatic juice, *trypsin*, *chymotrypsin*, and *carboxypolypeptidase*. The first two are secreted in their inactive forms, as *trypsinogen* and *chymotrypsinogen*. All three enzymes and the two zymogens have been isolated in crystalline form.

Enterokinase, an enzyme in the intestinal mucosa, activates trypsinogen; the trypsin thus formed in turn activates more trypsinogen, as well as chymotrypsinogen. Both enzymes act on native proteins, forming proteoses, peptones, and polypeptides. They also clot milk, but this property is possessed in a much higher degree by chymotrypsin than by trypsin. The polypeptides are acted on further by carboxypolypeptidase, the products being simpler peptides and amino acids.

Pancreatic Lipase, Steapsin. The fat-splitting enzyme of the pancreas, *steapsin*, is relatively inactive in the form in which it is secreted. However, in the presence of certain substances, such as bile, bile salts, egg albumin, calcium salts, and calcium soaps, the enzyme seems to be

[74] *J. Exptl. Med.*, **48**, 849 (1928).

activated. This type of activation is obviously non-specific. Willstätter [75] and his pupils have shown that these activating agents exert their effect on pancreatic lipase by providing a specially favorable adsorption condition for the contact of the water-soluble enzyme with its insoluble substrate, fat. This conception is a departure from the view, which until recently has been generally accepted, that the bile salts transform the inactive zymogen, steapsinogen, into steapsin, and that, in addition, the bile salts accelerate fat hydrolysis because of their co-ferment action toward the active steapsin. Glick and King [76] obtained a definite correlation between activating effect and the property of reducing surface tension in a study of the activation of lipase by various organic compounds. They found, moreover, that substances which activated lipase inhibited esterase and *vice versa*.[77]

Amylopsin and Other Enzymes. *Amylopsin* is the starch-splitting enzyme or amylase of the pancreatic juice. It is active in a neutral or slightly alkaline solution. The starchy food reaching the small intestine is digested by pancreatic amylase to the maltose stage. The maltose is hydrolyzed to glucose by the pancreatic and intestinal *maltases*. *Lactase* is not found uniformly in pancreatic tissue of adults, but occurs more consistently in children and other young mammals. This enzyme converts lactose into glucose and galactose. For the most part, the disaccharides are acted on by the intestinal enzymes. However, *invertase* is occasionally found in pancreatic juice.

The quantity of pancreatic juice is said to vary with the type of food, probably because of an interrelationship with gastric secretion. The secretion of the pancreatic juice begins when the acid chyme enters the duodenum, the quantity secreted being more or less conditioned by the amount of acid admitted. Cessation of pancreatic secretion in pathological conditions, as in obstruction of the pancreatic duct by a tumor, or in experimental occlusion of the pancreatic ducts by ligation, is usually followed by a reduction in the digestion of protein and fat. It has been stated (Yesko) [78] that under such conditions there is a delayed emptying time of the stomach which permits gastric digestion to proceed further than normally. Nevertheless, large amounts of material remain undigested and are found in the feces. This occurs, likewise, in animals after pancreatectomy. Even in these animals, with special care in the selection of the diet, fair nutrition may be maintained by virtue of the digestive powers of the gastric and intestinal secretions.

[75] *Z. physiol. Chem.*, **125**, 93 (1922–23).

[76] *J. Biol. Chem.*, **97**, 675 (1932).

[77] The blood contains an esterase, capable of splitting ethyl butyrate, but no true lipase is present. Following injury to the pancreas, or ligation of the pancreatic duct, the blood acquires fat-splitting properties. This may be interpreted as being due to the appearance either of a lipase or of a lipase-activating substance. A similar, though less pronounced, effect follows liver injury. I. S. Cherry and L. A. Crandall, *Am. J. Physiol.*, **100**, 266 (1932); *Proc. Soc. Exptl. Biol. Med.*, **28**, 570 (1931).

[78] *Am. J. Physiol.*, **86**, 483 (1928).

The Enzymes of the Intestinal Juice. Closely associated with the pancreatic juice in the digestive processes that occur in the intestines is the intestinal juice, or *succus entericus*. This secretion is produced most abundantly in the duodenum and is formed in progressively smaller quantities in the lower portions, the jejunum and the ileum. The juice, which is alkaline in reaction, is produced by two types of glands present in the mucous membrane of the entire small intestine, the so-called Brunner's and Lieberkühn's glands. There are apparently two distinct types of secretions, only one of which is associated with digestion. The other, which is periodic, occurring about every two hours even during starvation, is rich in the glycoprotein, mucin, and poor in enzymes, and contains a number of constituents which are very probably products of excretion. This periodic secretion and the bile form the major portion of the feces eliminated in starvation.

The discharge of food into the intestine is accompanied by the secretion of a juice which has moderate digestive properties; the greater digestive effects are produced in the mucosa itself. Two of the enzymes present in the intestinal juice carry forward, and practically to completion, the digestion of protein-split products. *Aminopolypeptidase* acts on polypeptides with the formation of amino acids and dipeptides. The dipeptides are in turn hydrolyzed by *dipeptidase* (p. 139). These enzymes were formerly known as "erepsin." A trypsin-like enzyme is said to be present in the intestinal mucosa. Here also occurs a *polynucleotidase* which converts nucleic acids into nucleotides. The latter are hydrolyzed by *nucleotidase* (phosphatase) into phosphoric acid and nucleosides. The purine nucleosides are hydrolyzed by *nucleosidase*. Mention has been made of the occurrence of *enterokinase* in the intestinal mucosa. There are also present *amylase*, *maltase*, *invertase*, and *lipase*, each acting on its respective substrate.

Cajori [79] has studied the digestive properties of intestinal juice from Thiry loops of the jejunum and ileum. He found the enzyme activity from jejunal loops much greater than from the ileal loops. Starch disappeared from the intestinal loops at a rate commensurate with the concentration of the amylase content of the juice, but the amount of peptone that was apparently hydrolyzed was much greater than could be accounted for on the basis of the "ereptic" activity of the juice. Sucrose and lactose were also absorbed more rapidly than was to be expected from the activities of invertase and lactase. When intestinal secretion was augmented as a result of histamine injections, there was no corresponding increase in enzymes. Lactase was encountered only once, the remaining samples being devoid of this enzyme. Although there have been occasional reports of the presence of lactase in intestinal juice, its occurrence in this fluid is not generally conceded.

Intestinal juice obtained from isolated loops of the intestine at various levels have been subjected to careful analysis in D. W. Wilson's

[79] *Am. J. Physiol.*, **104**, 659 (1933).

laboratory.[80] It was found that sodium, potassium, and calcium were relatively constant in all secretions, while the bicarbonate and chloride showed marked reciprocal variation. Ileal and colonic secretions contained proportionately more bicarbonate and less chloride than jejunal secretion and were accordingly more alkaline. In Table XXVI are selected data of the composition of fluid obtained from jejunal, ileal, and colonic secretions.

TABLE XXVI

ACID-BASE COMPOSITION OF INTESTINAL SECRETIONS

Type of Secretion	Base in Milliequivalents per Liter				Acid in Milliequivalents per Liter				
	Na	K	Ca	Total	Cl	HCO_3	PO_4	Total	*p*H
Jejunal.....	149	5 1	2.4	156 5	147	16 3	3 5	166 8	7.16
Ileal.....	148	4 7	5.5	158.2	87.9	97 3	.. ..	185.2	7.61
Colonic.....	151	8.6	5.0	164 6	87 5	91.8	.....	179 3	8.03

The Bile. The bile is continually formed by the liver cells and, between periods of digestion, is stored in the gall bladder. As it reaches the intestine, it is composed not only of the secretions of the liver cells, but likewise of the mucosa of the gall bladder and the biliary passages. The quantity of bile secreted is subject to great variation, and accurate determinations are not available. In man, the secretion for 24 hours has been estimated to be about 500–600 cc.

Bile is usually golden yellow, but may be brownish yellow or olive green in color. It is very bitter to the taste.

Bile obtained from the hepatic duct differs in composition from that found in the gall bladder chiefly with regard to the solid constituents, liver bile containing a much lower percentage of total solids. While in the gall bladder, the bile becomes concentrated by the reabsorption of a certain amount of water. Mucin and possibly other substances are added to the bile, being secreted from the wall of the gall bladder. Whereas gall bladder bile is nearly neutral, or even slightly acid (*p*H 5.4–6.9), hepatic duct bile is alkaline (*p*H 7.4–8.5).

It has been determined that the content of total base (Na + K + Ca) in hepatic bile is approximately equivalent to,[81] or is somewhat higher[82] than, that of the plasma, i.e., about 170 mM. per liter. The principal anions are chloride, bicarbonate, and bile-acid radicals. These show considerable individual variability in content, but in the aggregate approximate the total concentration of base. (Fig. 21.)

[80] E. J. de Beer, C. G. Johnston, and D. W. Wilson, *J. Biol. Chem.*, **108**, 113 (1935).

[81] J. L. Gamble and M. A. McIver, *J. Exptl. Med.*, **48**, 837 (1928); see also J. L. Gamble, *Bull. Johns Hopkins Hosp.*, **61**, 151 (1937).

[82] I. S., Ravdin, C. G. Johnston, *et. al.*, *Am. J. Physiol.*, **100**, 317 (1932).

In the gall bladder, there is reabsorption of water and of the bicarbonate and chloride ions. Indeed, the last may be reduced to an almost negligible quantity. However, the bile salts are not reabsorbed and hence increase in amount. There is likewise an increase in the amount of base, which not infrequently attains a level of more than 300 m.eq. Inasmuch as the base is largely in combination as bile salts, which are weakly dissociated, the osmotic pressure of gall-bladder bile is not much different from that of hepatic bile.[83]

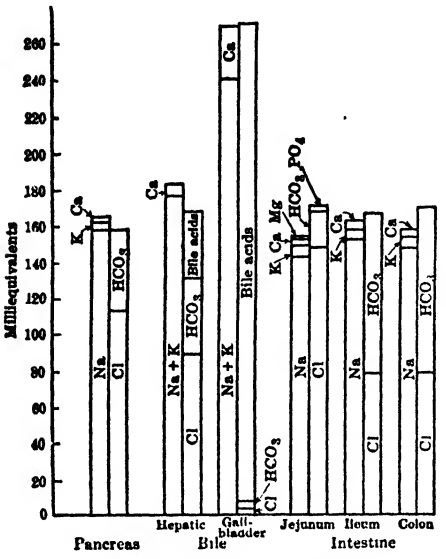


FIG. 21.—Composition of pancreatic juice, intestinal juice and bile. After J. P. Peters, "Body Water," C. C. Thomas, Springfield, Ill., 1935, p. 180. Based on data of E. G. Ball (pancreatic juice), *J. Biol. Chem.*, **86**, 433 (1930); Ravdin *et al.*, (bile), *Am. J. Physiol.*, **100**, 317 (1932); and de Beer, Johnston and Wilson (intestinal juice).[80]

There is also a marked increase in calcium and cholesterol. The absorption of chloride and bicarbonate and the non-absorption of calcium, bile pigment and cholesterol from the normal gall bladder have been demonstrated by Ravdin and associates.[84] In the diseased gall bladder, on the other hand, the normal relations are not found; substances which are ordinarily reabsorbed remain in the gall bladder.[85]

The approximate distribution of the chief constituents of human bile is indicated by the data in Table XXVII, based partly on the analyses of Hammarsten and v. Gorup-Besanez. The data are in parts per hundred.

Bile Pigments. The color of bile is due to the presence of a variety of pigments, chief among which is bilirubin, $C_{33}H_{36}N_4O_6$, a substance closely related to porphyrin (p. 244). On oxidation, this yields a green pigment, biliverdin, $C_{33}H_{36}N_4O_8$. The latter, on oxidation, forms a

[83] Gilman and Cowgill (*Am. J. Physiol.*, **104**, 476 [1933] have determined the osmotic pressure of blood, hepatic bile, pancreatic juice, and lymph collected simultaneously. All these fluids were found to be practically isotonic. When the osmotic pressure of the blood was temporarily raised or lowered, as by the injection of hypertonic saline, or water, corresponding changes occurred in the bile, pancreatic juice, and lymph. Evidently the secretions of the alimentary tract, though characteristic in composition, are nevertheless in osmotic equilibrium with the circulating fluid.

[84] Ravdin, Johnston, J. H. Austin, and C. Riegel, *Am. J. Physiol.*, **99**, 638, 648, 656 (1932); *J. Exptl. Med.*, **56**, 1 (1932).

[85] Johnston, Ravdin, Riegel and C. L. Allison, *J. Clin. Investigation*, **12**, 67 (1933).

number of compounds, among which is the blue pigment, bilicyanin. Bilicyanin does not occur in normal bile, but is found in gallstones, together with bilirubin, biliverdin, and other pigments In diarrhea, the feces may have a greenish color due to biliverdin.

TABLE XXVII

APPROXIMATE COMPOSITION OF HUMAN BILE*

	Hepatic Bile	Bladder Bile
Water	97.48	85 92
Solids	2.52	14.08
Specific gravity	1 010	1.040
Bile salts	0 93	9.14
Mucin and pigments	0 53	2.98
Cholesterol	0.06	0.26
Fatty acids and fat	0.14	0 32
Inorganic salts	0 84	0.65

* Hammarsten-Mandel, "Physiological Chemistry," p. 437, 1915.

It was formerly supposed that the bile pigments were formed exclusively in the liver. It now seems probable, however, that they may be formed in other tissues as well (spleen, bone marrow, lymph glands, etc.), and that the reticulo-endothelial system is principally involved in the process.

Bilirubin is derived mainly from the hemoglobin liberated in the process of red cell destruction. That this is not the only source has been suggested by Whipple and Robscheit-Robbins,[86] who consider muscle hemoglobin (myohemoglobin) as a precursor of bilirubin. Myohemoglobin and red cell hemoglobin are, however, not identical (p. 632). It has also been suggested that foods, notably proteins and green vegetables, may provide precursors for bilirubin (Whipple and Hooper).[87] The exogenous origin of bilirubin is not generally accepted.

After reaching the intestine, bilirubin is reduced to urobilinogen (stercobilinogen). This forms urobilin (stercobilin) [88] on oxidation. Both pigments occur in the feces normally.

A considerable part of the urobilinogen is absorbed in the portal circulation to be re-excreted in the bile. At most, only traces of urobilinogen normally reach the kidney. After the urine is voided the urobilinogen is converted into urobilin. Abnormal amounts of this pigment appear in the urine when the excretory function of the liver is severely impaired (action of poisons such as chloroform, severe hepatitis), or in hemolytic jaundice. In the last condition there is a marked increase in bilirubin production and therefore also of urobilinogen formation in the intestine. A proportion of the relatively excessive amount of the urobilinogen absorbed from the intestine fails to be re-excreted in the bile and therefore reaches the kidney to be eliminated in the urine. Accordingly the

[86] *Am. J. Physiol.*, **78**, 675 (1926).

[87] *Ibid.*, **40**, 349 (1916).

[88] For a recent study of the origin and identity of urobilin and stercobilin, see C. J. Watson, *J. Biol. Chem.*, **114**, 47 (1936).

urine in hemolytic jaundice frequently contains not only bile pigment, but urobilin as well. On the other hand, in complete biliary obstruction, bile pigment does not reach the intestine and therefore no urobilinogen is formed. Hence, as a rule the urine in obstructive jaundice contains bile pigment, but no urobilin (or urobilinogen).

Bilirubin gives a characteristic color reaction (red-violet) when treated with a mixture of sulfanilic acid, hydrochloric acid, and sodium nitrite. This is the well-known diazo reaction of Ehrlich. It has been adapted by van den Bergh and his associates to the detection of abnormal amounts of bile pigment in the serum, as well as to their quantitative estimation. The addition of a small amount of the diazo reagent to serum may result in the immediate development of a red-violet color, reaching its maximum intensity in 30 seconds. This has been described as the *direct reaction* and is indicative, clinically, of the obstructive type of jaundice.

No color may develop, or it may develop very slowly and incompletely, in a serum which after treatment with alcohol responds readily and completely. This is the *indirect reaction.* It has been associated clinically with non-obstructive, or hemolytic, jaundice.

Intermediate between the direct and indirect reactions is the *biphasic* reaction. In this type, the color develops as in the direct reaction immediately or within 30 seconds, but the maximum intensity is reached after a variable period.

What is the underlying cause for the difference in the direct and indirect reactions? This question has interested many investigators and has stimulated considerable discussion, but no definite explanation has yet been reached. The more generally accepted theory is that the bilirubin which normally circulates in the blood differs from the bilirubin that has passed through the liver cells in that the former is bound up in some manner with protein or lipids so that the bilirubin is prevented from reacting with the diazo reagent. After the complex is dissociated by alcohol, it is assumed, the reaction can take place.

Sodium bilirubinate gives a direct van den Bergh reaction. In an interesting series of experiments, Barron [89] added to serum increasing amounts of this pigment in a solution buffered to *p*H 8.43. The serum gave an indirect reaction until the concentration exceeded 12 mg. per 100 cc. As it increased to 16 mg., the reaction became biphasic. When the concentration exceeded this amount a direct reaction was obtained. Accordingly Barron suggests that " some constituent of the serum has a tendency to adsorb bilirubin, and this adsorption prevents coupling with the diazonium salt." [90]

[89] *Medicine,* **10**, 77 (1931).

[90] On the other hand, Griffiths has reported the isolation from gall-bladder bile of a pigment giving the direct van den Bergh reaction, which is distinct from bilirubin. The formula $C_{32}H_{50}O_{11}N_2$ is tentatively suggested, and the name *cholebilirubin* is proposed. *Biochem. J.*, **26**, 1155 (1932).

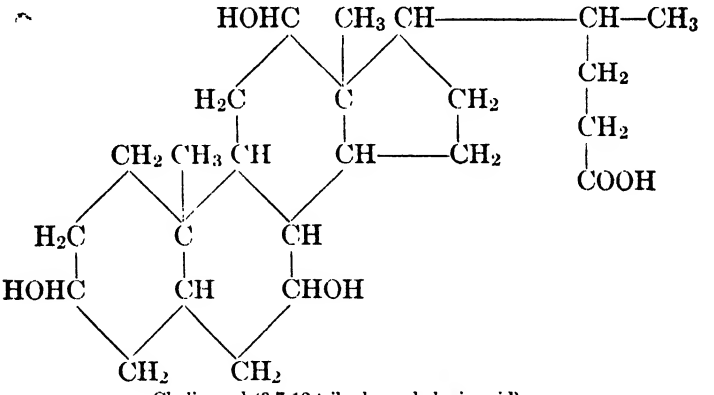
Bile Salts. Among the constituents of bile are the salts of the bile acids. Human bile contains glycocholic acid ($C_{26}H_{43}NO_6$) and taurocholic acid ($C_{26}H_{45}NSO_7$). In addition, glycocholeic acid ($C_{26}H_{43}NO_5$ or $C_{27}H_{45}NO_5$) has been detected in human bile, and, more recently, many new bile acids have been described. Glycocholeic is present in considerable amount in ox bile. Taurocholeic acid ($C_{26}H_{45}NSO_6$ or $C_{27}H_{47}NSO_6$) is present in dog bile and ox bile but has not been found in human bile. Hyoglycocholic acid ($C_{27}H_{43}NO_5$) occurs in the bile of pigs and chenotaurocholic acid ($C_{29}H_{49}NSO_6$) in the bile of geese.

On hydrolysis, glycocholic acid yields cholic acid and glycine, whereas taurocholic acid is converted into cholic acid and taurine (aminoethylsulfonic acid).

$$\underset{\text{Glycocholic acid}}{C_{23}H_{39}O_3\cdot CO\cdot HN\cdot CH_2\cdot COOH} + H_2O = \underset{\text{Cholic acid}}{C_{23}H_{39}O_3\cdot COOH} + \underset{\text{Glycine}}{CH_2NH_2\cdot COOH}$$

$$\underset{\text{Taurocholic acid}}{C_{23}H_{39}O_3\cdot CO\cdot HN\cdot CH_2CH_2SO_2\cdot OH} + H_2O = \underset{\text{Cholic acid}}{C_{23}H_{39}O_3COOH} + \underset{\text{Taurine}}{H_2N\cdot CH_2\cdot CH_2\cdot SO_2OH}$$

The structural relationship of cholic acid to cholesterol (p. 79) is shown by the following formula: [91]



Cholic acid (3,7,12,trihydroxycholanic acid)

The bile salts diminish the surface tension of the limiting membrane of red corpuscles and most other cells. In sufficient quantity they may exert a solvent effect on the cell lipids, causing the complete disintegration of the cells. To these properties of the bile salts Horrall and Carlson [92] have attributed the toxicity of bile when it leaves its normal

[91] For a full account of the chemical constitution of bile acids, consult L. Fieser, "The Chemistry of Natural Products Related to Phenanthrene," New York, 1936; Supplement, 1937. The reader is also referred to H. Sobotka, "Physiological Chemistry of the Bile," Williams & Wilkins, Baltimore, 1937.

[92] *Am. J. Physiol.*, **85**, 591 (1928).

channels, the biliary tract and alimentary canal, as in bile peritonitis and obstructive jaundice.

Injected intravenously into experimental animals, bile salts cause marked circulatory depression and eventually failure, muscular twitching, spasms, and other symptoms. The neuromuscular junction and the reflex centers of the cord are the structures most susceptible, according to the observations of Ries and Still.[93]

Functions of the Bile. Normal bile flow appears to be necessary for life; yet bile-fistula animals may tolerate the exclusion of bile over considerable periods. Especially is this true when the diet is carefully selected. Whereas fistula dogs kept on a diet of kitchen scraps usually die within two months, they may live in good condition for four to ten months when fed a diet of milk, cooked potatoes, rice, and bread. These animals usually develop bony abnormalities, however, the essential features of the condition being a loss of inorganic salts from the bones, which thus become thin and fragile. This has been attributed to failure in reabsorption of the calcium excreted in the intestinal juice.

Exclusion of the bile leads to serious digestive disturbances. The bile is a good emulsifying agent; it also promotes the solution of fats, fatty acids, and other lipids, and, according to the older view, exerts a direct effect in activating and accelerating lipases. Because of these properties the bile plays a very important rôle in the digestion and absorption of fats. Fat digestion is intimately associated with the digestion of other foodstuffs. The formation of a fatty layer around food particles diminishes the amount of surface exposed to the action of enzymes. As a result, in the absence of bile, a relatively large amount of undigested or partially digested food finds its way into the large intestine, where it is likely to undergo putrefactive changes. The bile ordinarily diminishes putrefaction by aiding in the digestion and absorption of fats as well as by its natural laxative properties. The bile stimulates peristalsis.

In his review of the extra-hepatic functions of the bile, Schmidt [94] has emphasized the importance of the bile as a reservoir for alkali. Together with the pancreatic and intestinal juices, bile neutralizes the hydrochloric acid which enters the intestines from the stomach. Owing to its recirculation, the bile affords a method of bringing alkali to the intestinal tract.

The bile is a channel for the elimination of certain excretory products —cholesterol, lecithin, drugs, toxins, bile pigments, and various inorganic substances.

Another function is the cholagogue effect of the bile acids. Bile taken internally stimulates biliary secretion.

Functions of the Gall Bladder. The sphincter of Oddi, a muscular band surrounding the common bile duct near its duodenal end, closes when digestion ceases. The continuous secretion of bile raises the pres-

[93] *Ibid.*, **91**, 609 (1930). [94] *Physiol. Rev.*, **7**, 129 (1927).

sure in the ducts, and, it is believed, after a certain pressure is reached, namely one of about 70 mm. of water, bile begins to flow into the gall bladder. Discharge of bile into the duodenum occurs when the pressure in the ducts rises above 100 to 120 mm., which is presumably the pressure maintained during digestion. Considerable variations in pressure in the bile passages have been observed under different conditions.

Not all animals have a gall bladder. The horse, certain deer, and the rat are among the species of animals in which it is absent.

As to the functions of the gall bladder, there are differences of opinion, as may be judged from various reviews of the subject.[95] The oldest and probably still the most widely accepted view is that the gall bladder serves as a reservoir during the intervals between digestion, when the bile is not needed. However, the gall bladder is not a reservoir in the same sense as the urinary bladder, for the bile which it can hold is only a portion of the total which enters the intestinal tract. In man the capacity of the gall bladder is approximately 3 per cent of the total daily bile flow. Actually, however, the importance of the gall bladder as a reservoir may be much greater than this figure would indicate, if due account is taken of the fact that the bladder bile is much more concentrated than hepatic bile.

The observation that the gall bladder is an organ of absorption of bile constituents has led many investigators to consider absorption as its main function, by virtue of which certain valuable materials, secreted in the bile, are restored to the organism.

On the other hand, various substances, such as mucus and toxic agents, are added to the bile during its stay in the gall bladder. Accordingly, it has been suggested that the gall bladder is secretory, and perhaps also excretory, in function.

The view has been advanced that the gall bladder is concerned in regulating the flow of bile, making possible an intermittent rather than a continuous flow into the intestine. Finally, there is the plausible theory that, because it is an expansible chamber, the gall bladder regulates the pressure in the biliary passages.

Both nervous and hormone mechanisms are said to control the flow of bile. The afferent nerve endings of the mucous membrane of the intestine are thought to be excited by the acid chyme when it enters the duodenum, resulting in a reflex contraction of the gall bladder and the flow of bile into the duodenum. Many workers have questioned the importance of this factor.

The hormone secretin, which, we have seen, stimulates the pancreas and intestinal glands, has been thought to act also on the liver cells, causing increased bile formation. However, Ivy and Oldberg[96] pre-

[95] See for example F. C. Mann, *Physiol. Rev.*, **4**, 251 (1924); B. Halpert, *Arch. Surgery*, **19**, 1037 (1929).

[96] *Am. J. Physiol.*, **86**, 599 (1928); see also A. C. Ivy, "Factors Concerned in the Evacuation of the Gall-bladder," *Physiol. Rev.*, **11**, 345 (1932).

pared an extract of the upper intestinal mucosa which when injected intravenously caused the contraction and evacuation of the gall bladder. The view has been advanced by these workers that when acid is injected into the duodenum something gets into the blood which causes the gall bladder (in cats, dogs, and guinea-pigs, but not in rabbits) to contract. The active principle, presumably a hormone, has been named "cholecystokinin."

The introduction of fat into the duodenum is said to stimulate a copious flow of bile.

Gallstones. Biliary concretions, or gallstones, are occasionally formed in the gall bladder, usually around some foreign body, injured epithelial cells, or bacteria. Although gallstones may contain a preponderance of one constituent, as in the cholesterol stones, or of two constituents, as in the calcium carbonate-bile pigment stones, they all contain small, although at times only minute, amounts of other substances. Fats, soaps, fatty acids, lecithin, mucin, copper, zinc, iron, and manganese are among the organic and inorganic substances which may be present. Strictly, therefore, there are no *pure* gallstones, but for purposes of classification, it is convenient to designate as such certain concretions which consist mainly of one substance. The cholesterol stones, for example, may contain as much as 98 per cent of pure cholesterol.[97] A convenient classification of gallstones has been proposed by Halpert.[98]

One species of whale (*Physeta macrocephalus*) develops biliary concretions containing a substance, ambrine, which closely resembles cholesterol. These concretions are often found in the excreta of these animals and are known as ambergris.

[97] For quantitative data of the composition of gallstones, consult T. W. Ray, *J. Biol. Chem.*, **111**, 689 (1935); see also A. J. Delario, *Am. J. Digestive Diseases, Nutrition*, **2**, 511 (1935–36).

[98] *Arch. Pathol.*, **6**, 623 (1928).

CHAPTER VIII

ABSORPTION AND INTESTINAL RESIDUES

The end-products of digestion diffuse through the wall of the small intestine, pass into the small blood and lymph vessels of the intestinal wall, and are then transported by the blood and lymph to the tissues. The undigested, unabsorbed residue is propelled to the large intestine and finally excreted as feces.

So much of the general plan is known. As to the precise mechanism involved in intestinal absorption, we are very much in the dark. Essentially, the problem is but one phase of the more general problem of cell permeability. Some of the more puzzling questions, pertaining to this subject, which we are as yet unable to answer satisfactorily, are set forth in the excellent review of Jacobs,[1] here quoted:

Beginning with the alimentary system, the problem of cell permeability arises in many forms. Why, for example, does practically no absorption, even of water, occur in the stomach, while taking place with the greatest ease in the small intestine? Why, in the latter, are some substances absorbed much more rapidly than others; for example, NaCl more rapidly than Na_2SO_4, dextrose more rapidly than sucrose, etc.? Why does NaCl readily enter the blood stream from a solution introduced into the gut but pass with difficulty in the reverse direction? Does the wall of the intestine show evidence of a one-sided permeability to water? What are the means by which water is taken up, not merely from hypotonic, but from isotonic and hypertonic solutions as well? What is the mechanism of normal absorption of the different kinds of digested food materials? . . .

Factors in Absorption. Among the more obvious factors which influence the amount of absorption from various parts of the alimentary canal may be mentioned the following:

1. Character of the lining epithelium.
2. Area of the absorbing surface.
3. Time during which food remains in contact with the absorbing surface in a particular region.
4. Amount of digested material present.

Absorption from the Upper Alimentary Tract. The epithelium of the mouth, pharynx, and esophagus is relatively impermeable and no food material is absorbed from these areas. Certain drugs, however,

[1] In Cowdry's "General Cytology," p. 99, 1924.

are absorbed, owing to their ready penetration and the vascularity of the tongue and the lining of the oral cavity.

There is, likewise, very little absorption from the stomach. While the gastric mucosa secretes large amounts of water, it normally absorbs but little or none. The gastric mucosa is somewhat permeable to alcohol and alcoholic solutions, as well as to very small amounts of sugar, amino acids, and other organic compounds. It is stated that condiments, such as mustard, increase the permeability of the gastric mucosa.

Absorption from the Small Intestine; Function of the Villi. The small intestine is best adapted for absorption, especially the lower part of the duodenum and the jejunum. Superficially, the surface of the small intestine measures about $\frac{1}{2}$ square meter, but the mucous coat is so irregular, because of its folds (*plicae circulares*) and its numerous smaller projections or *villi*, that the actual absorbing surface is about 10 square meters. Moreover, the food remains in the small intestine for several hours. It usually requires four to six hours, from the time the stomach begins to discharge its contents or acid chyme, before intestinal digestion and absorption are complete. The distribution of the chyme over so large an area as is offered by the small intestine greatly facilitates the absorption of diffusible substances.

The villi are of primary importance in absorption. They are small finger-like projections consisting largely of a framework of reticular tissue containing many leucocytes in its meshes. The lining is simple columnar epithelium, containing many goblet cells. In size, the villi may vary between 0.5 and 0.7 mm. In man, the villi number between four and five millions.

Two channels take part in the removal of material. In the center of each villus is the central lacteal which opens into a plexus of lymphatics lying in the muscularis mucosae. Fluid is forced from the lacteal toward the larger lymphatics by the contraction of muscle fibers which run lengthwise in the villus. The flow of fluid in the reverse direction is prevented by valves present in the deeper plexuses. After reaching the larger lymph vessels, the absorbed material, consisting largely of fat in emulsion, flows to the thoracic duct and enters the blood near the junction of the left subclavian with the jugular vein.

The capillary blood vessels of the villus constitute the second channel of absorption. The material diffusing into the capillaries is carried to the radicles of the portal vein and subsequently by the portal vein to the liver. The circulation of blood in capillaries is very rapid as compared with the sluggish flow of lymph. This is, no doubt, an important factor determining the distribution of material between the lymph stream and the blood.

Carbohydrate Absorption. Only the monosaccharides are readily absorbed. The disaccharides are not found in appreciable amount in the blood except when excessively large amounts are fed or when they are injected directly into the circulation. Under these conditions, sucrose

and lactose behave as foreign substances and are excreted as such by the kidneys. Maltose behaves somewhat similarly, although a certain amount is said to be transformed into glucose by a maltase present in the blood. Evidently the intestinal mucosa takes part in intracellular hydrolysis of disaccharides. No absorption of starch or dextrin occurs under similar conditions. The transformation of levulose and galactose to *d*-glucose has also been affirmed but is probably not complete when large amounts of these sugars are taken. The absorbed sugar is carried by the blood of the portal vein to the liver where much of it is removed and stored as glycogen. Other tissues, particularly muscle, likewise convert glucose into glycogen.

Sugar also enters the lymph circulation.[2] During the absorption of glucose, its concentration in the lymph and blood rises to about the same level. A marked increase in the concentration of sugar in the blood occurs soon after large amounts have been fed, but in the normal individual the blood sugar soon returns to normal levels even while absorption still continues. This shows that the liver and other tissues are capable of removing the sugar at a faster rate than it is absorbed. This capacity is markedly diminished in diabetes and in conditions in which the liver is involved.

In a quantitative study of carbohydrate absorption, Cori[3] found that sugars are removed from the intestine at a rate which is constant for each sugar and which is independent of the initial concentration of the sugar in the intestine. The following is the order of the rates of absorption of the sugars as determined by Cori: galactose > glucose > fructose > mannose > xylose > arabinose.

Groen[4] by a method of intestinal intubation studied the absorption of glucose, galactose, and fructose in man and found, as did others in experimental animals, that each sugar has its own individual absorption rate. The amount of sugar absorbed increases with an increase in intestinal surface, but is independent of the concentration, above a certain level (10 per cent for glucose). During absorption the concentrated sugar solution inside the intestine is rapidly diluted so that after one-half hour the total osmotic concentration of the intestinal contents equals that of the blood plasma.

These and other observations[5] indicate that intestinal absorption is not a simple process of diffusion. Substances, including sugars, in solutions of equimolecular concentration, when introduced into the intestine are absorbed at different speeds, which probably means that specific cellular mechanisms are involved. Evidence has been sub-

[2] *J. Biol. Chem.*, **32**, 299 (1917); M. Fay and P. S. Wharton, *ibid.*, **109**, 695 (1935); J. W. Heim, R. S. Thomson and F. C. Bartter, *Am. J. Physiol.*, **113**, 548 (1935).

[3] *Ibid.*, **66**, 691 (1925).

[4] *J. Clin. Investigation*, **16**, 245 (1937).

[5] H. E. Magee and E. Reid, *J. Physiol.*, **73**, 163 (1931); W. Wilbrandt and L. Laszt, *Biochem. Z.*, **259**, 398 (1933); E. Lundsgaard, *ibid.*, **264**, 209, 221 (1933); F. Verzár, *ibid.*, **276**, 17 (1935); H. B. Pierce, *J. Nutrition*, **10**, 689 (1935).

mitted that phosphorylation of glucose, fructose, and galactose occurs in the intestinal mucosa during their absorption. When the formation of esters is inhibited by certain agents (iodoacetate, phlorhizin), the rate of absorption is sharply reduced.

Fat Absorption. The possibility of the passage of unsplit fat across the intestinal wall cannot be overlooked entirely, but, as Bloor [6] has pointed out, there is every reason to believe that fat is completely hydrolyzed before it passes from the intestine. This view is opposed to the doctrine advanced by Munk more than forty years ago that neutral fat in fine emulsion is absorbed by the villous epithelium.

Pflüger [7] proposed the theory that fatty acids were not absorbed as such but as soaps. This conception was practically unchallenged by students of the subjects who differed with Munk, inasmuch as it was generally assumed that the intestinal contents were alkaline. Actually the reaction is almost always slightly acid. Even in the ileum the *p*H rarely rises above neutrality (McClendon,[8] Verzar [9]). Moreover, it has been shown that soaps dissociate at *p*H 8, and hence it is impossible for them to exist in the physiological range of *p*H 6–8.[10] The evidence therefore points strongly to the absorption of fatty acids as such.

Absorption of fatty acids is greatly facilitated by the presence of bile. Whereas the higher fatty acids are quite insoluble in water, they readily dissolve in an aqueous solution of bile acids. The property of the bile acids in bringing the otherwise insoluble fatty acids into solution in water has been described by Neuberg [11] as an example of *hydrotropism.* The effect seems to be due largely to the formation of bile acid-fatty acid complexes which are characterized by their diffusibility through membranes and by their stability in slightly acid solution. The bile acids, moreover, lower surface tension, thereby increasing the permeability of the epithelial cells and in this way probably promote the absorption of other substances as well.

Within the cell the bile acid-fatty acid complex is dissociated and the fatty acid released. Resynthesis of neutral fat occurs immediately and may be demonstrated histologically. How the fat is transferred from the lining epithelium into the lacteals is not clearly understood. According to the view of Heidenhain the fat globules are expelled from the epithelial cells by the contraction of the cell protoplasm. Shäfer considered the leukocytes to play the dominant rôle in the transfer of the fat to the lacteals. A third explanation is the hydrolysis-resynthesis theory of Loevenhart which is based on the reversible action of lipase.

[6] *Physiol. Rev.*, **2**, 92 (1922); see also R. G. Sinclair, *Ann. Rev. Biochem.*, **6**, 245 (1937).

[7] *Arch. ges. Physiol.*, **80**, 111 (1900); **82**, 303 (1900).

[8] *Am. J. Physiol.*, **38**, 191 (1915).

[9] *Nutrition Abstracts and Rev.*, **2**, 441 (1933).

[10] A Jarisch, *Biochem. Z.*, **134**, 163 (1923).

[11] *Ibid.*, **76**, 107 (1916); compare with M. H. Irwin, J. Weber, and H. Steenbock, *J. Nutrition*, **12**, 365 (1936).

According to this theory, the transfer of fat is made possible because of its hydrolysis at cell boundaries and its resynthesis within the cell. A critical examination of these rival theories leaves much to be desired. The problem of the transference of fat to the lacteals remains to be solved.

After entering the lacteals, the fat appears as a milk-white emulsion to which the term *chyle* has been applied. The chyle enters the larger lymphatics of the mesentery, passes to the receptaculum chyli, then by way of the left thoracic duct enters the blood at the junction of the left subclavian and jugular veins.

However, only about 60 per cent of the absorbed fat can be accounted for in the chyle. What happens to the remainder has always been somewhat of a mystery. A considerable portion is doubtless absorbed directly by the blood as shown by the higher concentration of fat in portal blood than in the general circulation. Eckstein [12] has studied the question of fat absorption through channels other than the left thoracic duct. While his results are admittedly not altogether consistent, they show, nevertheless, that when all the thoracic lymph is diverted from the blood stream, an appreciable, though small, augmentation of the fatty-acid content of the blood follows the absorption of neutral fat from the duodenum. It has been suggested, likewise, that a portion of the fat that is unaccounted for may be stored somewhere along the path of transport to the blood, or that it may be catabolized in the tissues before reaching the blood. In the blood, the fat is transported as neutral fat, fatty acid, and lecithin, and in the form of cholesterol esters. This question will be considered again in relation to the intermediary metabolism of fat.

The comparative rate of absorption of different fats has been investigated by Steenbock, Irwin, and Weber.[13] It was found that: (1) partially hydrogenated vegetable oils were absorbed as rapidly as lard or corn oil, and (2) that butter oil, halibut-liver oil, and cod-liver oil were absorbed uniformly at a more rapid rate than lard, corn oil, or the partially hydrogenated fats.

In a study of fat utilization in infancy, Holt and associates [14] found that absorption of a mixed fat is favored by the presence of (*a*) fatty acids containing one or more unsaturated linkages, and (*b*) fatty acids with relatively short carbon chains. Fat digestion and absorption are impaired in the premature infant.

Absorption of Proteins. The digestion of protein to amino acids serves many purposes. Except for minute amounts, the intestinal epithelium is normally impermeable to protein as well as to its intermediate digestion products—proteoses, peptones, and higher polypeptides.[15]

[12] *J. Biol. Chem.*, **62**, 737 (1925).
[13] *J. Nutrition*, **12**, 103 (1936).
[14] *J. Pediatrics*, **6**, 427 (1935).
[15] The absorption of unaltered or native protein, in sufficient amount to be detect-

For the most part, only amino acids are absorbed, although the simpler peptides are no doubt also diffusible through the intestinal epithelium. Were it not for this exclusion of nearly everything except the amino acids, much of the protein ingested would be of little use to the animal organism. To synthesize proteins characteristic of itself, the organism must begin with the simplest building-stones possible. The building specifications, so to speak, must be observed most rigidly, a difference in even a single peptide bond being sufficient to alter the architecture and properties of the protein molecule.

The amino acids are absorbed into the blood capillaries of the villi, the rate of absorption being somewhat different for indiyidual amino acids (Wilson and Lewis,[16] Höber and Höber [17]). Several hours after a meal, the amino-acid content of the blood, and especially of the corpuscles, is increased considerably. However, the blood is not the only channel of amino-acid absorption; evidence of absorption into the lacteals has been reported by Hendrix and Sweet,[2] who found the amino nitrogen of the chyle to increase considerably during absorption, becoming much greater in concentration than in the blood of the systemic circulation.

Not only would the absorption of proteins, proteoses, and peptones as such prove useless to the animal organism, but their entrance into the blood may be attended by a severe form of intoxication, termed "anaphylactic shock." Proteins differ in their toxicity and in the manner in which they act. This effect is especially pronounced for the proteoses and peptones and has been attributed to a variety of constitu-

able by immunological methods, has been amply demonstrated. For illustration may be cited the study of Sussman, Davidson, and Walzer (*Arch. Internal Med.*, **42**, 409 [1928]). In one series of 34 subjects they obtained evidence of egg protein absorption in 29. The absorption of fish proteins was also noted. In a later study of 62 children, ranging in age from 11 months to 4 years (*Am. J. Diseases of Children*, **50**, 49 [1935]), absorption of egg protein occurred in 45.

Ratner and Gruehl, in a comprehensive study of the passage of native proteins through the normal gastrointestinal wall (*J. Clin. Investigation*, **13**, 517 [1934]), found that about 50 per cent of guinea pigs (mature, as well as young animals), could be sensitized and shocked by the administration of protein foods. These results are considered evidence of the passage of unsplit protein (egg protein, milk protein, etc.) through the intact intestinal wall under normal conditions. This conclusion they believe to be equally valid for man, having found that proteins, natural, as well as foreign to the diet, may pass the intestinal wall and enter the blood stream.

In this connection, an interesting development is the report by Alexander, Shirley, and Allen (*J. Clin. Investigation*, **15**, 163 [1936]) that egg white, fed to dogs, is detectable in the systemic blood as well as in the lymph from the thoracic duct, but not in the portal blood, by various immunological procedures (precipitin tests, Prausnitz-Küstner technique of passive transfer, and by anaphylactic tests). On the basis of these observations it would follow that the route of ingested protein to the systemic circulation is *via* the thoracic duct lymph.

[16] *J. Biol. Chem.*, **84**, 511 (1929).

[17] *Proc. Soc. Exptl. Biol. Med.*, **34**, 486 (1936).

ents which may be supposed to arise during the hydrolysis of protein. It has been suggested that either histamine or substances related to it may be the fundamental cause of peptone shock.

Proteins, therefore, are foods when absorbed in the usual way as amino acids, and poisons when introduced directly into the blood. One of the most violent poisons known is ricin, the protein of the castor bean. The injection of a protein foreign to the tissues of an animal results in the excretion of most of it in the urine. If the injection is repeated a few days later, no ill effects ensue. Continued injection of small amounts of a given protein at short intervals establishes an immunity for that protein, due, it is believed, to the formation of a precipitin, in the presence of which the foreign protein is precipitated. If, however, the second injection is administered several weeks after the first, severe shock is induced. This phenomenon is termed *anaphylaxis* and has among its symptoms a marked fall in blood pressure and a reduction in the coagulability of the blood. According to some investigators anaphylactic shock and peptone shock are essentially the same, the former being due to the development in the sensitized animal of an enzyme capable of converting the foreign protein in question into proteoses and peptones. "Serum-sickness" frequently occurs in individuals sensitized against horse-serum proteins, and develops after the injection of antitoxins, such as diphtheria antitoxin. Under these conditions typical anaphylactic shock may occur and may terminate fatally.

Idiosyncrasies toward food proteins are very common. Certain individuals are unable to tolerate egg or milk proteins. Others, after eating strawberries or sea food, develop skin eruptions, asthma, and other anaphylactic or allergic reactions. These are attributed to the absorption of native or unchanged proteins found in these foods.[15] Exceedingly small amounts (less than one milligram) are frequently sufficient to produce typical reactions.

Occasionally, therefore, unchanged protein may be absorbed from the intestine. When this happens, the protein behaves as a foreign substance, or, where the individual has been previously sensitized to that protein, it behaves as a noxious agent. Ordinarily, however, protein is absorbed almost entirely in the form of amino acids.

Mechanism of Intestinal Absorption. In the present state of knowledge, we cannot speak with assurance concerning the forces involved in the absorptive process. No doubt, diffusion or osmotic forces play an important part, but there is the possibility that other factors are likewise involved.

Only when the intestinal epithelium is injured, as in poisoning with sodium fluoride, is it possible to demonstrate a definite relationship between osmotic forces and absorption. Dead intestinal epithelium behaves in many respects like an artificial gelatin membrane. Neither exhibits the characteristics of selective absorption shown by the living intestinal wall.

Cohnheim [18] studied the interchange of substances between the intestine and the circulating fluid in dead animals by pumping through the blood vessels a solution of sodium chloride (0.94 per cent). A sugar solution was placed in an isolated loop of the intestine, with the result that interchange of material occurred in both directions, sugar passing into the circulating fluid and sodium chloride into the intestine. There was no diminution in the volume of the intestinal contents. These observations have led many to the conclusion that absorption is due to some specific activity of the living epithelium.

The absorption of amino acids, glucose, sodium chloride, and similar substances from hypertonic solutions is easy to understand, but their absorption from isotonic or hypotonic solutions has always been somewhat of a puzzle. Why does absorption continue under these circumstances? It has been surmised that water is absorbed from isotonic or hypotonic solutions until the concentration in the intestinal contents again attains hypertonicity, when absorption is again possible, this process presumably continuing until all the solute is absorbed.

Starling [19] advanced the idea that the absorption of fluid from the intestines may be explained on the basis of the colloid osmotic pressure of the blood or lymph of the villi. This conception anticipated by many [redacted] later deductions based on the theory of membrane equilibria developed by Donnan. Starling's conception has received considerable support from the more recent work of Wells,[20] which indicates that the absorbing force of the intestine is proportional to and corresponds with the osmotic pressure exerted by the protein of the lymph in the lacteals. The colloid osmotic effect is due to protein derived from the plasma. The presence of protein in the lymph, according to Wells, causes an inward transfer of water, a process which apparently may continue indefinitely owing to the rapid transfer of the water to other tissues and the organs of excretion (kidney, lung, skin).

In a review of the subject, Magee [21] has considered the various factors (permeability, temperature, concentration of solute, diffusibility of the solute) which he believes should influence the rate and direction of the transfer of the solute through the intestinal wall, on the assumption that the rôle of the epithelial membrane in absorption is purely passive. In summarizing the evidence he states that the majority of the experimental results cannot be explained by known physical laws and that "the conclusion may therefore be drawn that the phenomena of absorption are due partly to some special property of the epithelial cells."

Formation of Feces. Under normal conditions the organic constituents of the diet are almost completely digested and absorbed before

[18] *Z. Biol.*, **37**, 443 (1899).

[19] E. H. Starling, "Fluids of the Body," London, 1909, p. 49; cited by Magee[21].

[20] *Am. J. Physiol.*, **99**, 209 (1931–32); **101**, 421. 434 (1932).

[21] *Physiol. Rev.*, **10**, 473 (1930). An earlier review of the literature is that of S. Goldschmidt, *ibid.*, **1**, 421 (1921).

reaching the lower part of the intestine, so that the dejecta from the ileum contain essentially the same quantities of nitrogen, fat, and carbohydrate as are present in normal stools (Welch, Wakefield, and Adams [22]). The inorganic composition of ileal contents is of interest, the observations in man being comparable to the analytical data obtained in the dog (p. 189). Welch and fellow-workers analyzed the dejecta from an ileostomy stoma of a patient and found the concentration of sodium to be 131–146 milliequivalents per liter of water. About 60 per cent was combined as chloride and the rest chiefly as bicarbonate. Taking into account the concentrations of other electrolytes (potassium, calcium, magnesium, phosphate), it becomes apparent that the fluid portion of ileal contents is in osmotic equilibrium with the blood.

The intestinal contents, upon reaching the ileocaecal valve, are not like feces in appearance and composition. They are semi-fluid in consistency, and frequently acid in reaction, whereas the feces are slightly alkaline. At this stage the intestinal contents consist largely of undigested food remnants, the remains of the digestive and intestinal secretions, and cellular elements, including cell débris from the alimentary tract. The transformation of this material into feces occurs in the large intestine where the food residues remain for one or more days. Here, certain substances, especially water, sodium [redacted], are reabsorbed often to a very marked degree. From the evidence submitted by Welch and associates [22] it does not seem that the colon has the function of excreting such substances as calcium and iron, as is often stated.

The character of the feces depends only partly on the food eaten. Thus, on a diet consisting exclusively of rice, the feces may have nearly the same composition as on an exclusively meat diet. The two foods, which differ in composition, are presumably almost completely digested and the feces are derived largely from the secretions of the alimentary tract. In a starving animal, the feces are diminished in amount, but the composition may be the same as in a normally fed animal. The feces are bulky when the food contains much indigestible material, like cellulose. Normally, the color is dark brown, but when much fat is present the stool acquires a characteristic lighter color. The composition of feces is about 60–70 per cent water, 5–10 per cent nitrogen, 10–20 per cent fatty material, and 10–20 per cent ash. According to Robinson [23] the normal fecal reaction of healthy men lies between *p*H 7 0–7 5.

Feces may contain the following food residues: cellulose, fruit seeds and skins (also made up largely of cellulose), muscle fibers, shreds of connective tissue, starch, fat, fatty acids, and soap. Among the remains of bile and intestinal secretions are to be found bile acids, bile pigments, cholesterol, coprosterol, mucin, and a variety of inorganic

[22] *Arch. Internal Med.*, **58**, 1095 (1936).
[23] *J. Biol. Chem.*, **52**, 445 (1922).

constituents, especially iron. Cellular elements derived from the alimentary tract are likewise present. One-fourth or more of the feces consists of bacteria, the number excreted per day having been estimated to vary between 50 and 500 billions.[24]

The fecal excretion of fat has been the subject of careful study. Hill and Bloor [25] and Sperry and Bloor [26] have shown that the amount and composition of fecal fat are to a large extent independent of the fat in the diet. In an experiment in which the effect of diet was studied, the amount of fecal fat on a fat-free diet was 1.76 grams, having an iodine number of 32.7. When this diet was supplemented with 50 grams of coconut oil, which has an iodine number of 8.8, the fat excretion was 2.50 grams, and its iodine number was 24.8. When 50 grams of olive oil (iodine number, 88.2) was added to the diet, the feces contained 2.24 grams of fat, having an iodine number of 44.6. Confirmatory evidence that fecal lipids do not represent a residue of the fat of the diet has been reported by Krakower,[27] who could find little, if any, change in the composition of the stools as a result of varying the kind and quantity (8 to 128 grams per day) of fat consumed.

Fecal fat, though differing in composition from food fat, resembles closely the lipids of the blood. Approximately one-third of the fecal lipids is unsaponifiable.[28] The endogenous fecal lipids do not originate, as might be suspected, from bacterial synthesis, desquamated epithelium, or other cellular débris of the intestines, but are for the most part secreted into the small intestine. A portion of this secretion is reabsorbed, apparently from the large intestine, but this is not certain. The unabsorbed residue, together with the relatively small amount secreted in the colon, make up the endogenous lipids found in the feces.[29]

In chronic idiopathic steatorrhea (non-tropical sprue, celiac disease, Gee's disease) the stools are very frequent and voluminous and contain large amounts of fat, fatty acids, and soaps. Accompanying the faulty utilization of these substances there is a loss of calcium, frequently of such proportion as to cause extensive demineralization of the skeleton. In advanced, untreated cases, the serum calcium may be depressed sufficiently to result in tetany.[30]

[24] For an account of the nature and composition of the feces, the student is referred to Chapter II of Lusk's "Science of Nutrition," 4th edition, Saunders, Philadelphia (1928).

[25] *J. Biol. Chem.*, **53**, 171 (1922).

[26] *Ibid.*, **60**, 261 (1924).

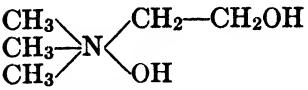
[27] *Am. J. Physiol.*, **107**, 49 (1934).

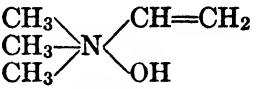
[28] *J. Biol. Chem.*, **68**, 357 (1926); **71**, 351 (1926–27); **81**, 299 (1929); **96**, 759 (1932); Sperry and R. W. Angevine, *ibid.*, **96**, 769 (1932).

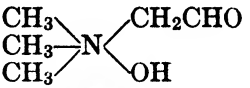
[29] See Shapiro, Koster, Rittenberg and Schoenheimer, *Am. J. Physiol.*, **117**, 525 (1936).

[30] A. M. Snell and J. D. Camp, *Arch. Internal Med.*, **53**, 615 (1934); E. Morgensen, *Quart. J. Med.*, **6**, 119 (1937).

Products of Intestinal Putrefaction.[31] The contents of the large intestine undergo bacterial or putrefactive changes. Concerning bacterial action on fat, little can be said except that it results in the formation of fatty acids and glycerol. From lecithin may be formed choline, neurine, muscarine, and related compounds.


Choline


Neurine


Muscarine

The carbohydrates yield a variety of substances, including oxalic acid, the lower fatty acids[32] and their derivatives—formic, acetic, propionic, lactic, butyric, oxybutyric, and succinic, acetone, and the gases carbon dioxide, methane, and hydrogen. If present in sufficient amount, some of the products of carbohydrate fermentation may act as irritants to the intestinal tract and cause diarrhea.

Bacterial enzymes acting on protein yield proteoses, peptones, amino acids, ammonia, and hydrogen sulfide. From the aromatic amino acids are formed indole, skatole, phenol, cresol, and tyramine. Cadaverine, putrescine, and ethylidenediamine are among the toxic amines, or ptomaines, formed from amino acids in putrefaction. Ethyl mercaptan (C_2H_5SH), methyl mercaptan (CH_3SH), and hydrogen sulfide owe their origin to the putrefaction of cystine.

With regard to the formation of these substances, much remains to be learned, but sufficient is known to enable us to consider briefly the chemistry of some of the reactions involved. Among the more important of these is one involving the removal of a carboxyl group (decarboxylation), presumably due to an enzyme, carboxylase, present in the bacteria. Another reaction consists in the splitting off of an amino group by deaminization. Reduction, due to a reductase, and reactions of hydrolysis as well as of oxidation are also believed to occur.

Tyramine is formed in the putrefaction of cheese and is known to be a constituent of certain cheeses—Camembert, Roquefort, Emmenthal, etc. It is also a constituent of ergot. It has likewise been isolated from intestinal contents. Tyramine is a pressor base (raises blood pressure) but is weaker in its action than epinephrine (p. 486), to which it is closely related chemically. Tyramine is detoxified in the liver, being at least partly converted into hydroxyphenylacetic acid.

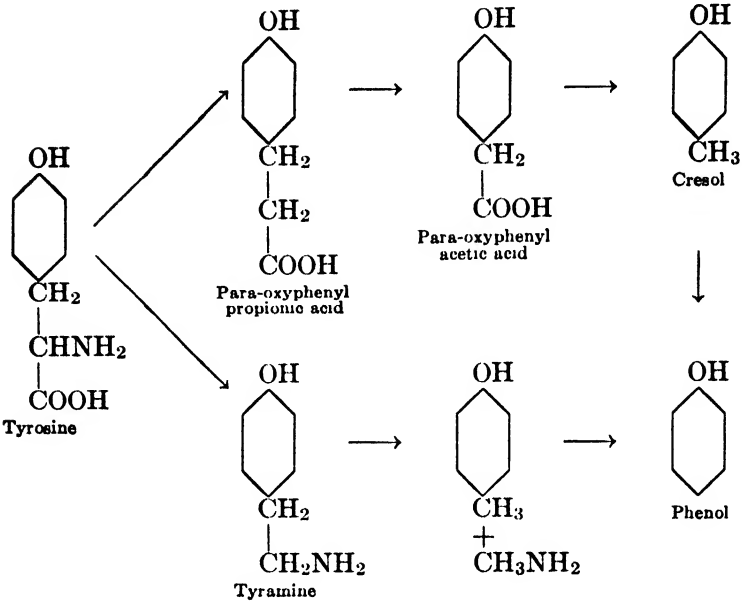
Hanke and Koessler[33] in an examination of 26 stools found 17 to contain a microörganism capable of decarboxylating tyrosine.

[31] For a comprehensive survey of the subject, the student is referred to Marjory Stephenson's monograph "Bacterial Metabolism," Longmans, Green & Co., London, 1930; see also *Ann. Rev. Biochem.*, **3**, 519 (1934); **4**, 593 (1935).

[32] Grove, Olmsted and Koenig, *J. Biol. Chem.*, **85**, 127 (1929–30).

[33] *J. Biol. Chem.*, **59**, 835 (1924).

The following substances result from bacterial action on tyrosine:



Cresol, phenol, and probably phenylacetic acid, after absorption, are partly conjugated with sulfuric acid and glycuronic acid (with the latter especially in herbivorous animals). Folin and Denis,[34] as well as Dubin,[35] have shown that 30–90 per cent of the phenols (this term is here applied to phenol and its derivatives) are excreted in the urine in the free form, the total amount eliminated usually varying between 200 and 400 mg. per day. Quantitatively, paracresol is most important.

The process of conjugation of the phenols with sulfuric acid, which takes place in the intestine primarily but in other organs as well, is a mechanism which the organism employs in detoxifying relatively toxic substances.[36] The fate of foreign organic compounds in the animal organism has been discussed by Sherwin [37] and will be referred to again.

Indole and Skatole. The disagreeable and characteristic odor of feces is said to be due partly to two compounds, indole and skatole. These

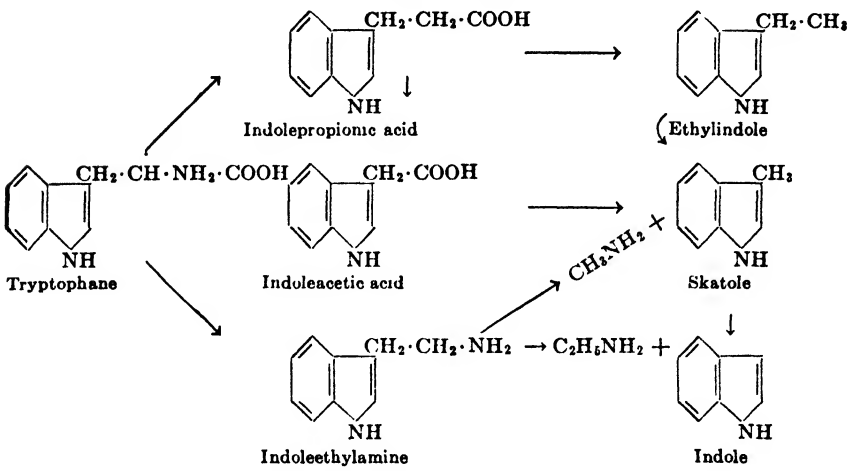
[34] *J. Biol. Chem.*, **22**, 309 (1915).

[35] *Ibid.*, **26**, 69 (1916); **31**, 255 (1917).

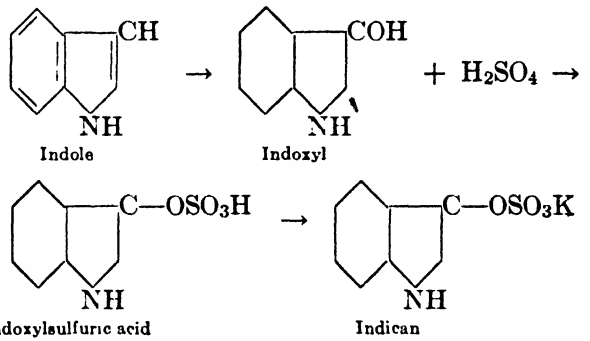
[36] For a recent discussion of the various kinds of detoxication mechanisms the reader is referred to the review by A. J. Quick, *Ann Rev. Biochem.*, **6**, 291 (1937); see also B. A. Houssay, *Am. J. Med. Sci.*, **192**, 615 (1936), for a good discussion of the origin and fate of phenols and of indoxyl.

[37] *Physiol. Rev.*, **2**, 238 (1922); see also A. M. Ambrose and C. P. Sherwin, *Ann. Rev. Biochem.*, **2**, 377 (1933).

are formed from tryptophane, as indicated by the following formulas:



A portion of the indole is oxidized either before or after absorption and is subsequently conjugated to form the potassium salt of indoxylsulfuric acid (indican), in which form it is excreted in the urine. The daily elimination of indican varies considerably, but is usually between 12 and 20 mg. The indole content of the feces averages about 50–60 mg. on an ordinary diet. In carcinoma of the liver, large amounts of indican appear in the urine. Indicanuria is present in a large proportion of patients affected with duodenal ulcer, achylia gastrica, and "toxic" headache (Soper [38]).

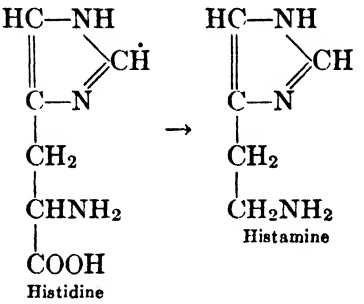


As much as 1 gram of indole may be administered to a dog without producing unusual symptoms. Larger amounts (2 grams) produce diarrhea and hematuria. The quantities normally absorbed from the intestine are probably insufficient to produce any effect in man. Very large amounts, however, are said to produce torpor, feeble heart action, and

[38] *Am. J. Digestive Diseases Nutrition*, **3**, 564 (1936–37).

lower temperature. Skatole behaves very much like indole but is somewhat less toxic. The amount of this substance normally excreted in the urine is less than 10 mg.

Histamine. Histamine (β-iminazolylethylamine) was first isolated from ergot by Barger and Dale.[39] Its formation through the action of putrefactive bacteria on histidine was first described by Ackermann.[40] It has been further shown by other investigators that a bacillus is present in the intestinal contents which is capable of decarboxylating histidine. Indeed, Hanke and Koessler [41] in an examination of 26 human stools found 16 to contain such a microörganism. The formation of histamine may be represented as follows:



Histamine has been isolated from intestinal contents, as well as from loops of the large and small intestine. Human feces contain appreciable amounts. Hanke and Koessler found 500 to 600 grams of feces from normal individuals to yield 6 to 20 mg. of histamine.

In variable amounts the presence of histamine has been reported in different organs (lung, muscle, heart, spleen, and intestinal and gastric mucosa). Its reported identity with the gastric hormone has been considered elsewhere (p. 172).

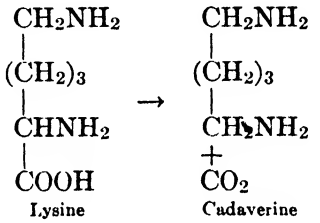
When injected intravenously, it is toxic, even in small amounts, producing, particularly in anesthetized animals, a condition of shock, simulating traumatic or surgical shock. However, when given by mouth, it is relatively inert, large amounts being tolerated. The histamine disappears. Its detoxication by the animal organism has aroused considerable interest. Unlike tyramine it is not destroyed by perfusion through the liver. It is, however, rendered physiologically inert by incubation with minced beef, lung, or kidney. The effect is due to an enzyme, histaminase, described by Best.[42] This is present in abundance, not only in lung and kidney, but also in intestinal mucosa, spleen, muscle, adrenals, and blood. Little, if any, is present in the liver and gastric

[39] *J. Physiol.*, **40** (Proc.), xxxviii (1910); **41**, 499 (1911).
[40] *Z. physiol. Chem.*, **65**, 504 (1910).
[41] *J. Biol. Chem.*, **59**, 879, 889 (1924).
[42] *J. Physiol.*, **67**, 256 (1929); Best and E. W. McHenry, *ibid.*, **70**, 349 (1930); *Physiol. Rev.*, **11**, 371 (1931).

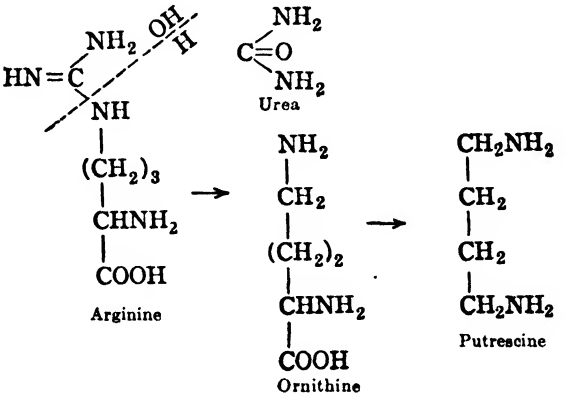
mucosa. Best and McHenry have suggested that the histaminase of intestinal mucosa may serve as a protection against the small amounts which normally might be formed. It is therefore doubtful that histamine formed in intestinal putrefaction can be a factor in systemic intoxications.

Cadaverine and Putrescine. Ptomaines is the term that has been applied to the basic substances derived from putrefying flesh. As we have seen in the case of tyramine and histamine they are formed from amino acids by the removal of a carboxyl group.

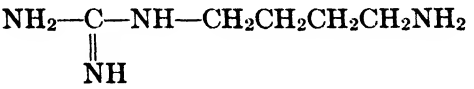
Of the more familiar ptomaines, one is cadaverine, a diamine (pentamethylenediamine), formed from lysine and first discovered in putrefying human cadavers, whence the name. Its formation may be represented as follows:



A closely related substance is putrescine (tetramethylenediamine) derived from arginine.

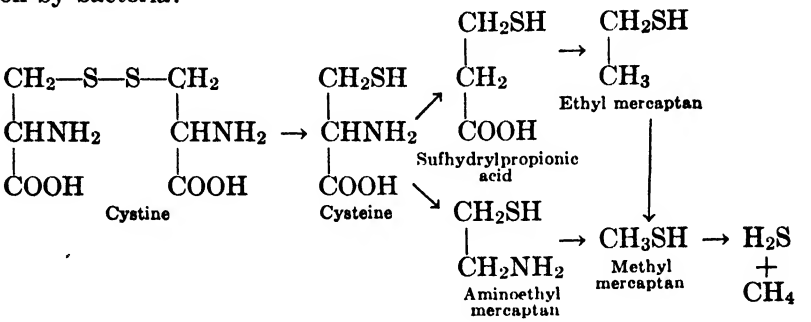


Another product formed in the putrefaction of arginine is agmatine,



Cadaverine and putrescine have been found in the urine in cholera.

Mercaptans. Cystine undergoes the following changes when acted upon by bacteria:



It is a popular belief that the absorption of products of intestinal putrefaction is responsible for many ills, as well as for the symptoms associated with constipation—headache, malaise, irritability, nausea, insomnia, drowsiness, etc. Actually, there is very little experimental evidence to support this view, as has been pointed out by Alvarez in reviewing the literature on the subject of intestinal auto-intoxication. Very little absorption takes place from the colon, especially when the feces are hard. If auto-intoxication were due to the absorption of toxic substances from the colon it would be more frequent in diarrhea than otherwise. Alvarez [43] ascribes the symptoms of constipation to the mere plugging of the lower end of the alimentary canal. A plug of cotton introduced into the rectum produces the same effect. He has also observed that individuals with jejunal fistulas become sleepy when the intestine is made to contract on a small balloon inserted through the fistula. Muscular activity and nervous stimuli arising in the digestive tract are probably important factors in producing the symptoms ordinarily associated with constipation.

Intestinal Obstruction. Occlusion of the intestine results in severe toxic symptoms, anorexia, weakness, profound depression, oliguria, continuous vomiting, sometimes muscular twitching and tetany, etc. The rapid onset, severity, and usually fatal outcome have led to two views as to the cause of the toxemia, one being that it is due to a toxic agent formed above the point of obstruction and the other that the noxious substance has its origin in the mucosa of the intestine or stomach. However, a closer study of the problem has revealed totally different factors to which the symptoms may be attributed. In the first place, it seems that the intestinal contents in obstruction are not more toxic than normal intestinal contents. Then it has been observed that in pyloric obstruction, or in obstruction of the duodenum or upper ileum, there are definite chemical changes in the blood. These include a

[43] *Physiol. Rev.*, **4**, 352 (1924); see also W. C. Alvarez, "The Mechanics of the Digestive Tract," 2d edition, Hoeber, New York, 1928.

striking fall in the concentration of chloride, a marked increase in the alkali reserve (carbon dioxide-combining capacity), and a terminal increase in non-protein nitrogen. Considerable evidence has accumulated in the last few years to indicate that the depletion of chloride and water, due to the copious vomiting, and the accompanying derangement of the water and acid-base balance are chiefly responsible for the symptoms in acute intestinal obstruction and possibly are also factors in other conditions where excessive loss of gastric juice occurs.

A crucial experiment demonstrating the effects of total loss of gastric juice is that of Dragstedt and Ellis.[44] These workers isolated the stomach of dogs by section at the cardia and pylorus. The duodenum was anastomosed to the lower end of the esophagus. The cardiac end of the isolated stomach was closed and the pyloric end brought to the surface as a fistula. Precautions were taken not to interfere with the vagus nerves or blood vessels supplying the isolated stomach. The total loss of the gastric secretion which was drained away through the fistula resulted in symptoms of weakness, anorexia, loss of weight (chiefly because of anhydremia), oliguria, and profound depression. Death occurred in five to eight days. Accompanying these symptoms and proportionate to their intensity, the following changes were noted in the blood: decrease in concentration of chloride, the values ranging between 340 and 108 mg. per 100 cc., an increase of the CO_2-combining capacity, reaching a value as high as 140 cc., an increase in *p*H (7.3–7.75), and a terminal increase in non-protein nitrogen. The changes in the blood chemistry thus resulting from the loss of gastric juice were therefore similar to those occurring in pyloric or intestinal obstruction. It was observed, moreover, that the gastric glands continued to secrete a juice of high acidity even when the blood chloride was reduced to less than one-third of its normal concentration. Dragstedt was able to relieve the symptoms, restore the blood constituents toward the normal, and prolong the lives of the animals (in one case to over 76 days) simply by intravenous injection of Ringer's solution. It was therefore concluded that, although food deprivation was one of the main effects of this experimental procedure, the symptoms and fatal outcome could be attributed to hypochloremia, alkalosis, and dehydration.

Scott, Holinger, and Ivy[45] have described an experiment on a dog having a pouch of the stomach and a jejunal fistula. Through the fistula the dog was given an adequate diet and sufficient salt (6 to 10 gm. daily) to maintain the normal level of blood chloride. Despite the complete loss of gastric juice (400 to 600 cc. daily), the animal continued in good health and maintained its normal weight for the period of the experiment (18 weeks).

The theory that the absorption of a toxin is a factor in the toxemia observed in intestinal obstruction is not supported by the experiments

[44] *Am. J Physiol.*, **90**, 331 (1929); **93**, 407 (1930).
[45] *Proc. Soc. Exptl. Biol. Med.*, **28**, 569 (1930–31).

of White and Fender,[46] who produced high intestinal obstruction in animals and kept them alive by the simple expedient of reinjecting the vomitus below the point of obstruction through a jejunal fistula. Jenkins [47] produced high intestinal obstruction in dogs and succeeded in prolonging their lives by short-circuiting the biliary, pancreatic, and duodenal secretions below the site of occlusion.

Loss of Pancreatic Juice. The loss of pancreatic juice leads to as conspicuous effects as the loss of gastric juice. Dogs with pancreatic fistulas invariably die within a short time (15 to 42 days in Pavlov's experiments;[48] 7 to 8 days in Elman and McCaughan's experiments).[49] Gamble and McIver [50] have definitely associated the symptoms and fatal outcome with the loss of water and electrolytes. Inasmuch as there is a relatively greater loss of basic (chiefly Na^+) than of acid radicals (chiefly Cl^- and HCO^-_3), acidosis develops. Thus Dragstedt and associates [51] have observed a reduction in total plasma base from 155 to 130 mM., a decrease in chloride from 111 to 84 mM., a decrease in HCO_3 from 23.0 to 8.3 mM., and a shift in the *p*H from 7.35 to 6.95. In short, the loss of either gastric or pancreatic juice results in dehydration. In the former case, depletion of the chloride ion predominates; hence alkalosis develops. In the latter case, there is a predominant loss of base; hence there is a shift of the acid-base balance in the blood to the acid side (see p. 269).

The loss of bile by continuous drainage may be expected to produce changes similar to those obtained when pancreatic juice is drained away.[52]

[46] *Arch. Surgery*, **20**, 897 (1930).
[47] *Ibid.*, **19**, 1072 (1929); **25**, 849 (1932); **26**, 407 (1933).
[48] "The Work of the Digestive Glands," translation by Thompson, London, 1902.
[49] *J. Exptl. Med.*, **45**, 561 (1927).
[50] *Ibid.*, **48**, 859 (1928).
[51] *Proc. Soc. Exptl. Biol. Med.*, **28**, 110 (1930–31).
[52] Herrin (*J. Biol. Chem.*, **108**, 547 [1935]) has reported that in dogs with a Thiry fistula secretion of considerable amounts of intestinal juice may be stimulated by distending the fistula with a rubber balloon. Continued loss of the secretion produces a decrease in serum chloride, bicarbonate, and fixed base, as well as a decrease in plasma volume due to anhydremia, and a corresponding increase in serum protein, calcium, and inorganic phosphorus. There is also a marked decline in the oxygen saturation of the blood. Accompanying the chemical changes in the blood which result from the loss of electrolytes in the intestinal juice, the animals develop the characteristic signs and symptoms of intestinal obstruction.

CHAPTER IX

THE BLOOD AND OTHER BODY FLUIDS

The interchange and transport of material in the animal organism are accomplished through the blood, lymph, and the interstitial or tissue fluid. The blood circulates in what is practically a closed system of vessels (the vascular system), and although there is an exchange of material through the endothelium lining the capillaries, the blood itself does not come in direct contact with the cells of the tissues, except in the liver and spleen, where in part the endothelium is non-continuous in some of the blood spaces. It is now believed by many physiologists and anatomists that the lymphatics also constitute a closed system of vessels and that they do not open directly into the tissue spaces as was formerly supposed. In the tissue spaces and bathing the cells is a fluid, the composition of which is supposed to resemble that of the lymph circulating in the lymphatics and which, according to Starling's theory, is derived from the blood by a process of passive filtration. The tissue fluid plays an important rôle in the exchange of material between the blood and tissues. Nutritive substances, including oxygen, brought by the blood pass through it on their way to the tissues, and, in turn, the metabolic products of the cells are carried into the blood and lymph streams through the reabsorption of the tissue fluid.

Functions of the Blood. Among the more important functions of the blood are the following:

1. It is concerned with the transportation of oxygen from the lungs to the tissues and of carbon dioxide from the tissues to the lungs. This constitutes the respiratory function of the blood.

2. The blood transports food material and other substances absorbed from the intestine to the tissues.

3. The waste products formed in metabolism are carried by the blood to the organs of excretion—kidneys, lungs, intestine, and skin.

4. The blood is the channel for the exchange of products formed in one tissue or organ and used by another. The hormones are transported in the blood.

5. The white corpuscles of the blood constitute a defense mechanism against invading bacteria. Other defenses of the body against toxic agents include antitoxins, agglutinins, precipitins, etc., contained in the blood.

6. The blood takes part in maintaining the temperature of the body at a fairly constant level.

7. It aids in the regulation of the normal reaction, or acid-base balance, of the tissues.

8. Owing to the plasma proteins, it is concerned in the regulation of the water balance of the tissues and of the body as a whole.

Volume. Various methods have been employed in attempts to determine the total volume of blood in the body. The direct procedure of measuring all the blood that could be removed by bleeding and subsequent extraction of the tissues has been used in experiments on animals and in executed criminals. Another method has been based on the property of carbon monoxide of reacting with oxyhemoglobin, replacing the oxygen and forming carbon monoxide hemoglobin. The amount of carbon monoxide thus combined may be determined quantitatively, and, the amount of the gas inhaled being known, the amount of blood may be estimated by appropriate calculations. Dyestuffs, such as congo red and vital red, have also been introduced for this purpose (Keith, Rowntree and Geraghty [1]). A definite amount of the dye is injected intravenously and after a short interval a specimen of blood is collected and centrifuged, the proportion of cells measured, and the concentration of dye in the plasma determined. On the assumption that no dye is taken up by the cells or escapes from the blood, the blood volume is calculated. Similar in principle to the dye method is the procedure of Lee and Whipple [2] in which a solution of hemoglobin is injected.

Results reported by different workers have been very divergent. The discussion of Fleischer-Hansen [3] calls attention to the technical errors of the exsanguination method. In the other methods the fundamental error is made of assuming that the ratio of cells to plasma is the same in all parts of the body. In consequence, the carbon monoxide method, though giving a reasonably accurate measure of the body hemoglobin (and volume of red cells), affords no accurate information concerning plasma volume. Likewise, the dye- or hemoglobin-injection methods give at best an accurate measure of the circulating blood plasma only, as shown by Whipple and associates.[4] More dependable information should therefore be obtained, according to these investigators, by a combination of the two methods, a small allowance being made for the volume occupied by the white blood cells.

The quantity of blood in the body is thus estimated to be about 8.8–9.2 per cent, or between one-eleventh and one-twelfth, of the body weight. It is apparently not subject to much fluctuation, physiologically or otherwise. Dehydration occurs particularly in infancy and childhood, due to water deprivation, diarrhea, or vomiting, and is accompanied by a concentration of the blood and a reduction in its volume.

[1] *Arch. Internal Med.*, **16**, 547 (1915); see also J. G. Gibson and W. A. Evans, *J. Clin. Investigation*, **16**, 301, 317, 851 (1937).

[2] *Am. J. Physiol.*, **56**, 328 (1921).

[3] *Skand. Arch. Physiol.*, **59**, 243 (1930).

[4] *Am. J. Physiol.*, **56**, 313, 336 (1921).

The blood volume is also diminished during severe and rapid hemorrhage and in surgical shock. It is increased in polycythemia vera, in fever, and during pregnancy. The cellular elements of the blood become more concentrated in influenza, following the inhalation of war gases, following severe burns, and intestinal obstruction. The term anhydremia has been applied to the condition in which the concentration of the blood is greater than normal. For a review of the literature on the subject, the student is referred to the article by Marriott.[5]

Formed Elements; The Red Blood Cell. The formed elements are the red corpuscles or erythrocytes, the white corpuscles or leukocytes, and the blood platelets. Together these constitute about 40–45 per cent by volume of the whole blood, the remainder being occupied by the plasma. In men, there are normally 5,000,000 red cells per cubic millimeter of blood; the count is somewhat lower in women. Variations occur frequently, especially after exercise or a heavy meal, or at high altitudes. The shape of the mammalian corpuscle is commonly that of a circular, non-nucleated, biconcave disk. The average diameter usually given for the red cells of man is 7.7 μ, a value obtained by examining dried preparations of blood and considered by Ponder[6] to be too low. Ponder's own measurements, made on red cells in the fresh state, show the human corpuscle to have an average diameter of about 8.8 μ and a range of variation from 7.5 to 9.5 μ. When circulating in the blood vessels, the red cell does not maintain a fixed shape but changes its form continually, especially in the small capillaries.

The average volume of a single red cell is approximately 85 cubic micra and the range of variation is normally 82–92 cu. μ. In certain forms of anemia the cells are abnormally large, hence their designation as macrocytic anemias, of which pernicious anemia is the most familiar form. In other kinds of anemia, the red-cell volumes may be abnormally small (average volume < 80 cu. μ). Normally the average hemoglobin content per cell is 29$\gamma\gamma$ (micro-micrograms; $1\gamma\gamma = 1 \times 10^{-12}$ gram). The average concentration per unit volume (mean corpuscular hemoglobin concentration) is 34 per cent.[7]

Loss of blood through hemorrhage, or an abnormally rapid destruction of red cells as often occurs in certain infections and intoxication with chemical hemolytic agents, unless fully compensated by increased blood production, leads to the development of anemia. However, anemia may also result from defective blood formation. Toxic agents, such as X-rays, benzol, or bacterial toxins, may so affect the bone marrow as to depress its activity. Or the diet may be so deficient in iron

[5] *Physiol. Rev.*, **3**, 275 (1923).

[6] E. Ponder, "The Mammalian Red Cell and the Properties of Haemolytic Systems," Berlin, 1934, p. 16.

[7] For additional details the reader is referred to a review by M. M. Wintrobe, "The Erythrocyte in Man," *Medicine*, **9**, 195 (1930); see also *Arch. Internal Med.*, **54**, 256 (1934).

that the erythrocytes which are produced contain less than the normal amount of hemoglobin. The most interesting group of anemias are those resulting from a lack of either one, or both, of two essential factors, the so-called *intrinsic* factor present in normal gastric mucosa and the *extrinsic* factor which occurs in certain foods (beef muscle, eggs, autolyzed yeast, rice polishings, wheat germ, etc.). The complex formed by the combination of these two factors is the *antianemic* principle. It is stored in the liver and acts on the bone marrow in promoting and hastening the maturation of the red cells. The intrinsic factor is lacking in the gastric mucosa of persons with pernicious anemia. The success first achieved by Minot [8] and associates in the treatment of pernicious anemia patients with liver and liver extracts has led to a fuller comprehension of the problems connected with the hematopoietic function.

The red blood corpuscles are continually undergoing destruction, new cells being formed to replace them. Most estimates place the average life of red cells at 30 to 40 days, but Wiener [9] considers 3 to 4 months to be a closer approximation. On the basis of the latter estimate, the number of red cells destroyed per hour is in the neighborhood of 10 billions. Preceding destruction, changes in the composition of the cells are believed to occur which render them less resistant. To a large extent, the corpuscles are taken up by the phagocytic cells of the reticuloendothelial system, such as the Kupffer cells of the liver. In the process of dissolution, the lipids of the membrane are dissolved, and the hemoglobin which is liberated is the most important, though probably not the only, source of bilirubin. Formerly the liver was thought to be the only site of red-cell disintegration. This view is no longer generally held, for it seems that the destruction of corpuscles may occur in the blood stream, as well as in other tissues.

During rapid red-cell disintegration and in certain diseases of the blood-forming system various types of nucleated erythrocytes (normoblasts, microblasts, macroblasts, megaloblasts) may appear in the circulation. The presence of reticulocytes (immature red cells showing a reticulum under vital staining) is likewise an indication of marked erythropoesis. Normally less than 0.5 per cent of the red blood cells are reticulocytes.

Hemolysis. Erythrocytes undergo *hemolysis*, or laking, when blood is diluted with water or treated with ether, chloroform, soap, fatty acids, ultraviolet rays, bile acids, saponin, snake venom, specific hemolysins, and other agents. Sometimes partial hemolysis is due to the disruption of erythrocytes by purely mechanical influences. When placed in a hypotonic solution, the red corpuscle swells, thus stretching the membrane. If the stretching is sufficient, the membrane bursts. There is some reason to believe that the distended membrane is more permeable

[8] G. R. Minot and W. P. Murphy, *J. Am. Med. Assoc.*, **87**, 470 (1926); see also W. B. Castle, *Harvey Lectures*, Series **30**, 37 (1934-35).

[9] *J. Am. Med. Assoc.*, **102**, 1779 (1934).

than the normal membrane. Under these conditions, the hemoglobin may pass out of the corpuscle into the plasma. However, it does not seem that the red cell undergoes complete destruction at once, for there remain behind the so-called " ghosts." From their appearance, it seems possible that the " ghosts " are made up of the stroma, or network, of the original corpuscles. The disintegration of the stroma, or *stromatolysis*, probably follows hemolysis when red corpuscles are subjected to the action of hemolytic agents.

Clinical application has been made of the resistance of erythrocytes to hypotonic solutions (fragility test). In concentrations higher than 0.42–0.44 per cent sodium chloride, human erythrocytes resist hemolysis at ordinary temperatures for as long as two hours. Beginning hemolysis is shown at about 0.40 per cent, but in more hypotonic solutions (0.32–0.36) hemolysis is carried to completion. Diminished resistance (increased fragility) is observed in hemolytic jaundice. Increased resistance seems to be a characteristic of the red blood cells in various forms of anemia.

The Leukocytes or White Blood Cells. The *leukocytes*, of which there are several forms (granulocytes—neutrophile, eosinophile, basophile; lymphocytes and monocytes) usually number between 5000 and 10,000. Wide fluctuations occur physiologically, but when the subject is at complete rest, the white blood cell count tends to be at a minimum and relatively steady. Food intake is thought to produce an increase (digestive leukocytosis), but it has been shown that if food is taken in the resting state, leukocytosis does not occur. Inflammation, and bacterial infection, in general, are usually accompanied by a sharp rise in the white count. The type of response varies. In most septic infections, the increase may be almost exclusively due to neutrophiles, whereas in tuberculosis and whooping-cough there is a relative increase in lymphocytes. There may also be a reduction in white cells (leukopenia), as in typhoid fever, and it is even possible for one type of cell to diminish or practically disappear from the circulation (granulopenia).

In a large measure the function of the leukocytes in the destruction of bacteria and in the liquefaction and removal of dead tissue is due to the various enzymes which they contain, particularly proteases and lipases.

Normally the platelets number 250,000 to 400,000. Increases up to a million and more have been reported in Hodgkins' disease and in myeloid leukemia. The most pronounced reduction, and even disappearance, of platelets occurs in *purpura hemorrhagica*, a condition in which spontaneous bleeding occurs from the mucous membranes and subcutaneously. The platelets are of biochemical interest because they contain the phospholipid, cephalin (p. 75), which as we shall see is a requisite in the process of blood clotting.

Serum, Plasma. If blood, after being drawn from the blood vessels, is allowed to stand, it soon forms a clot in which the cellular ele-

ments are enmeshed. Clot formation is due essentially to the conversion of the soluble protein, fibrinogen, into the insoluble protein, fibrin. Calcium is needed for this process. If the effect of the calcium is removed by its combination with an oxalate or citrate radical, the blood remains fluid. Blood treated in this way is referred to as *oxalated* or *citrated.*

Blood may also be kept from clotting by adding *heparin,* the function of which will be considered presently (p. 227).

The formed elements may be separated from the plasma by centrifuging the whole blood.

When allowed to stand, a blood clot eventually retracts and shrinks; in the process of shrinking, a pale yellow liquid, the serum, is expressed. A similar phenomenon is exhibited by other colloidal gels and is termed *syneresis.* Serum is blood from which the corpuscles and fibrinogen have been removed. Plasma is blood from which the corpuscles have been removed; it differs from serum in that it contains fibrinogen.

The fibrin may be removed in the form of stringy masses, without enmeshing the cellular elements, by rapidly stirring freshly drawn blood with a rod or some other contrivance. As a result *defibrinated* or "*whipped*" blood is obtained.

Composition. A discussion of the chemical constituents of the blood without reference to their physiological significance would have little meaning. The composition of the blood is so intimately related to its diverse functions that it is only logical to consider the various compounds individually and each from several angles.

Certain of the blood constituents have specific, though not necessarily limited, functions. Whereas fibrinogen is almost exclusively concerned in the clotting process, another blood protein, hemoglobin, not only has the specific function of transporting oxygen, but is also an important participant in the buffer mechanism of the blood and indirectly in the transportation of carbon dioxide. Similarly, the plasma proteins, inorganic salts, cell lipids, etc., are each connected with several properties of the blood.

Then there are those constituents which are primarily of nutrient value to the organism and may be regarded as being *en route* from the intestine, liver, or other organ to some tissue for utilization. Amino acids, glucose, fats, oxygen, and inorganic salts belong to this category.

A third group of constituents may be considered as waste products of metabolism, in transit from the tissues to the organs of excretion. Blood contains approximately 45 to 65 volumes per cent of carbon dioxide. The total non-protein nitrogen of the blood, which, as the term implies, represents all the nitrogenous constituents of the blood, except the proteins, varies normally between 25 and 35 mg. per 100 cc. The amino acids, which are not waste products, account for a certain proportion, but about 14 to 20 mg., or roughly 50 to 60 per cent, represents urea nitrogen. The amount of urea in the blood is therefore approximately 30 to 40 mg. per 100 cc. Blood of normal individuals contains 2 to 3 mg. of uric acid, 1 to 2 mg. of creatinine, 3 to 6 mg. of creatine

(which is not to be regarded as a waste product necessarily), an exceedingly small amount of ammonia (about 0.1 mg. per 100 cc.) and small amounts of other nitrogenous constituents, comprising the " undetermined nitrogen " fraction.

The chemistry of the blood is of much importance in relation to intermediary metabolism and the functional activity of the organs of excretion, notably the kidneys. In diabetes, owing to a derangement in carbohydrate metabolism, the sugar in the blood may increase considerably above normal. When fat oxidation is not carried to completion, such substances as acetone, acetoacetic acid, and β-hydroxybutyric acid, normally present in insignificant amounts, appear in the blood (and urine) in relatively large quantity. More than normal amounts of uric acid are formed and excreted when there is excessive metabolism of glandular material. Retention of the non-protein nitrogenous constituents of the blood may occur in disease of the kidney. The detailed study of certain of the blood constituents, such as urea, creatine, creatinine, uric acid, etc., will be deferred at this time for the reason that their significance will be more fully appreciated after their origin has been considered in the chapters on intermediary metabolism.

Specific Gravity. The specific gravity of whole blood is normally between 1.054 and 1.060. The plasma has a specific gravity of $1.026 \pm .002$.[10] For erythrocytes the value is approximately 1.090.

Water. Quantitively water is the most important constituent. The plasma (or serum) of mammalian blood, including that of man, contains 90–92 per cent of water; the red corpuscles, 64–65 per cent.[11]

[10] The specific gravity of the plasma corresponds to the protein content. When the latter exceeds 7.5 grams per cent, the specific gravity is correspondingly high, 1.028; and with values above 8 grams, it may be about 1.030. On the contrary a protein content of 6 grams per cent is associated with a specific gravity of about 1.024; 5 grams with 1.022; 4 grams with 1.019. See p. 223 for formula relating the protein concentration to the specific gravity of the plasma.

[11] Eisenman, Mackenzie and Peters (*J. Biol. Chem*, **116**, 33 [1936]) have determined the relations of water and protein in serum and cells of human blood. For serum the following equation approximates the relation most closely:

$$W_s = 98.5 - 0.745P_s$$

in which W_s is the cubic centimeters of water in 100 cc. of serum; P_s is the per cent of protein, and 0.745 is the specific molecular volume of the serum protein. The formula gives falsely high values for W_s in sera containing unusually large amounts of lipids.

The relation of hemoglobin to water in human red corpuscles is defined by either of the following equations:

$$(a)\quad W_c = 94.53 - 0.704Hb_c$$

$$(b)\quad W_c = 96 - 0.75Hb_c$$

in which W_c is the cubic centimeters of water in 100 cc. of cells and Hb_c is the per cent hemoglobin. Equation (*a*) was derived empirically from the analytical results; in equation (*b*) use was made of the approximate specific molecular volume of hemoglobin.

The relation of the total protein to water in the red cells is defined by the equation:

$$W_c = 100.93 - 0.887P_c$$

The similarity in composition of human, dog, and horse blood is shown by the data in Table XXVIII.

TABLE XXVIII

SPECIFIC GRAVITY AND WATER CONTENT, IN PERCENTAGE BY WEIGHT, OF WHOLE BLOOD, SERUM, AND RED BLOOD CELLS IN DOG, MAN, AND HORSE*

	Whole Blood		Serum		Cells	
	Sp. Gr.	H_2O	Sp. Gr.	H_2O	Sp. Gr.	H_2O
Dog	1.054	79.2	1.021	92 2	1 100	64 6
Horse	1.0548	80 93	1 028	91 39	.	63 8
Man	1 055	79 1	1 027	90 7	1 090	64 8

* The data for dog blood are based on the analyses of Austin, Cullen, Gram, and Robinson (*J. Biol. Chem.*, **61**, 829 [1924]), with the exception of the value for the specific gravity of the cells. The data for horse blood are based on the analyses of Van Slyke, Wu, and McLean (*J. Biol. Chem.*, **56**, 765 [1923]). The data for human blood and dog blood cells are the author's and are in good agreement with data to be found in the literature. See, for example, L. J. Henderson, "Blood, A Study in General Physiology," Chapter VI, Yale Univ. Press, New Haven, 1928.

Solids. The solid constituents of the human red blood corpuscle comprise approximately 35 per cent of the weight and may be roughly distributed as follows:

Hemoglobin	31–33 per cent
Phospholipids, cholesterol, and other lipids	1 "
Protein*	0.5–1 "
Inorganic constituents, ionized or combined, chiefly potassium and chloride	0.5–0.6 "
Organic constituents, glucose, glutathione, urea, etc.	0.2 "

* Globulin, globin, and nucleoprotein have been described as constituents of the stroma or red cell membrane, but according to E. Jorpes, *Biochem. J.*, **26**, 1488 (1932), the protein of the stroma differs from these, as well as from hemoglobin and fibrin.

Blood plasma contains 8–9 per cent solids, which are distributed approximately as follows:

Plasma protein	7 ± 0.5 per cent
Phospholipids, cholesterol, and other lipids	0.7 "
Inorganic constituents	0.75 "
Organic constituents, such as glucose, urea, amino acids, etc.	0.15 "

PLASMA PROTEINS

An accurate method for determining total protein in plasma or serum consists in separating the protein from the lipids by precipitation in acetone, and subsequent coagulation with acetic acid (and removal of contaminants), drying, and weighing.[12]

[12] C. O. Guillaumin, R. Wohl, and M. L. Laurencin, *Bull. soc. chim. biol.*, **11**, 387 (1929). See also Peters and Van Slyke, "Quantitative Clinical Chemistry," Vol. II, p. 688, Williams & Wilkins, Baltimore, 1932, for various methods for the determination of plasma protein.

Probably the most widely used procedure is the well-known Kjeldahl method for nitrogen, both in its original macro form and in the micro form, of which there are several variants. From the value for total nitrogen, the protein nitrogen is obtained by subtracting the non-protein nitrogen. There is also a colorimetric method based on the reaction of Folin and Denis' so-called phenol reagent with the tyrosine of the protein molecule. Several modifications of this indirect procedure have been described. Physical methods of measurement, employing the interferometer, viscosimeter, and refractometer, have been introduced, but these have not proved to be entirely reliable. Finally, the apparent relationship of the specific gravity of plasma to its protein content shows possibilities of wider application.[13] The relationship is expressed by the following formula:

$$\text{Total protein} = (\text{specific gravity} - 1.007)\ 343$$

The normal variation of the total protein of plasma is indicated by the following data:

TABLE XXIX

PLASMA PROTEIN CONTENT IN NORMAL INDIVIDUALS, IN GRAMS PER 100 CC.

Authority	Number of Subjects	Minimum	Maximum	Average
Salvesen [14]	32 (16 men and 16 women, 42 analyses)	6 34	7 96	7 00
Moore and Van Slyke [15]	9	6 5	7 7	7 1

The plasma proteins may be separated into three major fractions:

1. Fibrinogen.
2. Globulin.
3. Albumin.

Fibrinogen and the Clotting of Blood. Fibrinogen resembles the globulins; indeed it may be classed with them. Like the globulins it is precipitated by half saturation with ammonium sulfate; it differs in being precipitated in a 0.75 molar solution of sodium sulfate and by half saturation with sodium chloride. This property may be utilized in its quantitative estimation, but a more accurate procedure consists in diluting the plasma with sodium chloride solution, and adding a certain

[13] N. S. Moore and D. D. Van Slyke, *J. Clin. Investigation*, **8**, 337 (1929–30); A. A. Weech, C. E. Snelling, and E. Goettsch, *ibid.*, **12**, 193 (1933)

[14] *Acta Med. Scand.*, **65**, 147 (1926); cited by Moore and Van Slyke.

[15] *Loc. cit.*; see also Linder, Lundsgaard, and Van Slyke, *J. Exptl. Med.*, **39**, 887 (1924); Bruckman, d'Esopo, and Peters, *J. Clin. Investigation*, **8**, 577 (1929–30).

amount of calcium chloride. The fibrinogen is thus converted into fibrin, which may be separated, dried, and weighed. Or the nitrogen may be determined by the Kjeldahl method and from the result the amount of fibrinogen calculated.[16]

In human plasma the amount of fibrinogen varies between 0.2 and 0.4 per cent, though somewhat higher values are not uncommon. The following data are illustrative:

TABLE XXX

FIBRINOGEN PERCENTAGE IN PLASMA OF NORMAL INDIVIDUALS

Authority	Number of Subjects	Minimum	Maximum	Average
Gram [17]	25 (men)	0 20	0 36	0 27
	25 (women)	0 21	0 38	0.29
McLester [18]	15	0 272	0 385	0 333

The maintenance of the normal quantity of fibrinogen in the blood depends upon the integrity of the liver. Destruction of liver tissue, whether by disease or through the action of such poisons as chloroform, phosphorus, or hydrazine, leads to an impairment in the mechanism for the production of fibrinogen and results in a sharp fall of its content in the blood. On the contrary, in sufficiently small doses, these liver poisons may exert an irritant or stimulating effect on the liver parenchyma and cause a definite rise in the amount of blood fibrinogen (Foster and Whipple).[19]

Direct evidence of the rôle of the liver in the production of fibrinogen has been obtained in experiments on animals (rabbits, dogs) in which the liver had been completely extirpated. Such animals may be kept alive for many hours after the operation, provided the sugar content of the blood is maintained at its normal level, or somewhat higher, which may be done by the administration of glucose. In hepatectomized animals a progressive decrease of blood fibrinogen occurs invariably. A disappearance of 20 to 50 per cent has been observed in dogs (Jones and Smith) [20] within 12 to 20 hours. In rabbits, the rate of disappearance has been found to be even more rapid (Drury and McMaster),[21] but it

[16] G. E. Cullen and D. D. Van Slyke, *J. Biol. Chem.*, **41**, 587 (1920); see also T. B. Jones and H. P. Smith, *Am. J. Physiol.*, **94**, 144 (1930), and Peters and Van Slyke, Vol. II, p. 696.

[17] *J. Biol. Chem.*, **49**, 279 (1921).

[18] *J. Am. Med. Assoc.*, **79**, 17 (1922).

[19] Foster and Whipple (*Am. J. Physiol.*, **58**, 407 [1921–22]) obtained the following data for fibrinogen in 13 dogs, maintained on a liberal mixed diet; minumum 0.306 per cent; maximum 0.506 per cent; average 0.390 per cent. Lower values—average 0.358 per cent—were observed in fasting animals.

[20] *Am. J. Physiol.*, **94**, 144 (1930).

[21] *J. Exptl. Med.*, **50**, 569 (1929).

is of interest to note that, in animals in which the liver had been only partly removed (70 per cent), the blood fibrinogen remained unchanged. The depletion of fibrinogen in liverless animals is clearly an index of the rapidity of its normal utilization by the organism.

Equally remarkable is the capacity of the organism to regenerate fibrinogen. In studying this problem several investigators have employed the procedure of replacing the blood of an animal with its own defibrinated blood, or with the defibrinated blood of compatible donors. Drury and McMaster found that after a 90 per cent reduction of fibrinogen by this procedure (in rabbits), a complete return to the previous amount occurred within 5 to 6 hours. Indeed an enormous overproduction of fibrinogen ensued in the animals thus stimulated to regeneration of this protein. No such regeneration occurred, however, in hepatectomized animals subjected to the same defibrination procedure (Drury and McMaster, Jones and Smith). Not only was there no new formation of fibrinogen, such as occurred in the normal animals, but a swift fall in the amount of the substance signified a rapid utilization of the fibrinogen remaining in the organism. As emphasized by Drury and McMaster, had there been any other important source of fibrinogen, regeneration should have been observed in the liverless animals. Moreover, the body is apparently without any great reserve of fibrinogen, for otherwise the speedy decrease of this substance in the blood would have been prevented. In short, there is no evidence of fibrinogen regeneration in the absence of the liver. Consequently it is to be assumed that this organ either controls its production, or is actually the site of its formation.

Physiological and Pathological Variations. Foster and Whipple have reported that diets rich in animal protein (meat, liver, beef, heart) favor a high blood fibrinogen level, as contrasted with fasting, or carbohydrate or fat feeding. According to Vars,[22] this effect may be produced by any kind of protein, including casein, but the dietary stimulus to increased fibrinogen formation is transient and the high fibrin level cannot be maintained indefinitely.

The problem of fibrinogen utilization has not been studied adequately, but such knowledge as has been gained in recent years points to its participation, not only in disease, but normally, in the reparative processes of tissues and organs and particularly in the repair of injuries to the vascular system. Tissue injury and inflammation stimulate an increase in fibrinogen production; indeed, Foster and Whipple regard tissue injury as the most powerful single stimulus to overproduction of fibrinogen, a stimulus which far exceeds that of a low fibrin level such as results from severe hemorrhage. Moreover, these investigators have shown that bacteria are not directly concerned in the reaction, which is identical whether an inflammation is sterile or septic. Under these conditions there is an exaggerated utilization of fibrinogen in the for-

[22] *Am. J. Physiol.*, **93**, 554 (1930).

mation of fibrinous exudate and in the deposition of fibrin in and about the inflamed or injured tissues.

That almost any kind of infection or inflammation in the body leads to an increase in blood fibrinogen is supported by clinical observations. The following are data reported by McLester:[23]

TABLE XXXI

	Fibrinogen, Milligrams per 100 cc.		
	Minimum	Maximum	Average
Septic inflammation; various forms, (20 patients)	624	1120	829
convalescent, for instance after drainage of abscess (4 patients)			540
Pneumonia (8 patients)	726	1447	1069

Menstruation and pregnancy are likewise associated with a rise in plasma fibrinogen. In acute hepatitis the blood fibrinogen is elevated, but in the more severe forms of liver disease (acute yellow atrophy, phosphorus poisoning, etc.), low values are the rule.

The injection of proteose or peptone intravenously causes a severe and usually fatal intoxication, which is not accompanied by an inflammatory reaction, but by a marked destruction of tissue protein and a reduction in fibrinogen. The disappearance of this constituent is attributed to two factors (Foster and Whipple): a passive escape with other blood constituents from the blood vessels; an active escape due to precipitation of fibrin in certain areas injured by the proteose.

Clotting or Coagulation of Blood. Two consecutive reactions are believed to occur in the process of blood coagulation, as was stated by Hammarsten a half century ago.

1. Formation of thrombin from its precursors.
2. Conversion of fibrinogen into fibrin under the influence of thrombin.

Clotting does not occur in the absence of *ionizable* calcium, as may be shown by removing it through precipitation as the insoluble oxalate, or conversion into citrate. The function of the calcium has been explained in various ways.[24] According to Howell's theory, it is

[23] *J. Am. Med. Assoc.*, **79**, 17 (1922); *Arch. Internal Med.*, **35**, 177 (1925).

[24] Ferguson has observed that decalcification of newly formed thrombin is accompanied by loss of activity, whereas if the thrombin is allowed to "age" for several hours, subsequent decalcification does not affect its property of clotting blood. It may be supposed that the calcium participates in the formation of a calcium-containing intermediate complex which changes to stable thrombin. *Am. J. Physiol.*, **119**, 755 (1937); see also Editorial, *J. Am. Med. Assoc.*, **109**, 1818 (1937).

responsible for the formation of thrombin from prothrombin.[25]

$$\text{Prothrombin} \xrightarrow{Ca^{++}} \text{thrombin}$$

It is known that blood in contact with dead or injured tissue, or with disintegrated blood corpuscles, and especially with broken-down blood platelets, clots more readily than otherwise. Howell suggested that when blood platelets or tissue cells disintegrate a substance is liberated which hastens the coagulation process. This substance was originally named *thromboplastin*, but as it was found to be soluble in lipoid solvents and because of other properties it was concluded that it was either cephalin, or cephalin in combination with another compound, perhaps protein. The function of the cephalin in the clotting process has never been established with certainty, but it is believed by Howell [26] that it neutralizes the effect of *heparin*, a constituent of the blood which in some way prevents the transformation of *prothrombin* into *thrombin*. Heparin has been prepared from the liver (and other organs) and is believed to be responsible for maintaining the normal fluidity of the blood. Formerly this alleged effect of heparin on prothrombin was attributed to an hypothetical substance termed *antiprothrombin*. Another suggestion that has been advanced is that heparin combines with some other substance to form *antithrombin* and that this too is neutralized by cephalin. It is to be admitted that our conception of the process of clotting is not necessarily clarified by the introduction of so many terms to represent alleged substances, or rather effects.

In short, according to Howell's theory there are found in the blood fibrinogen, prothrombin, calcium salts, cephalin, heparin, and antithrombin. When blood is shed, owing to the disintegration of the blood platelets, or in some other fashion, cephalin or a cephalin compound is liberated. This combines with heparin and possibly antithrombin, with the result that prothrombin is formed and in the presence of the calcium salts is converted into thrombin.[27] The thrombin then transforms the fibrinogen into fibrin.

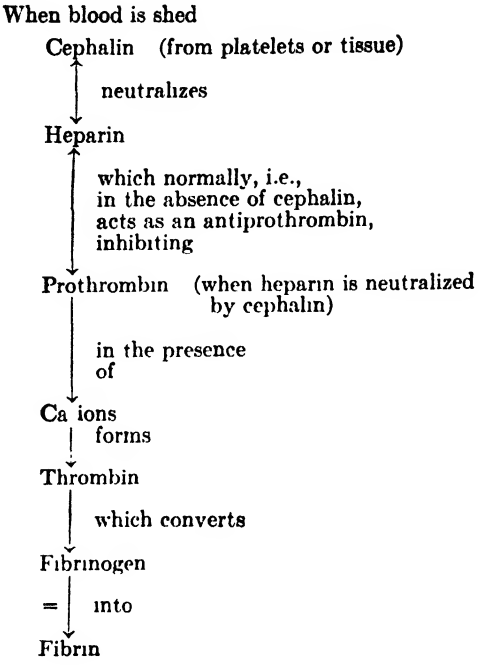
[25] *Am. J. Physiol.*, **29**, 187 (1911–12); **31**, 1 (1912–13); J. McLean, *ibid.*, **41**, 250 (1916); **43**, 586 (1917).

[26] *Bull. Johns Hopkins Hosp.*, **42**, 199 (1928).

[27] Quick has reported (*Am. J. Physiol.*, **115**, 317 [1936]) that heparin has only a slight inhibitory action on thrombin when tested with purified fibrinogen, in marked contrast to the strong anticlotting action it produces in plasma. He has therefore stated that heparin itself is not an antithrombin, although it apparently forms antithrombin by reacting with some constituent of the plasma. Quick has also denied the neutralization of heparin by thromboplastin (platelet derivative—cephalin). His conclusion is that the latter antagonizes the anticlotting action of heparin by accelerating the conversion of prothrombin to thrombin. The true antagonist of thrombin is therefore the inhibitory substance heparin produces in plasma.

The isolation of heparin has been reported by A. Schmitz and A. Fischer, *Z. physiol. Chem.*, **216**, 264 (1933). They consider it to be a monobasic acid of the formula $C_{18}H_{28}O_{17} \cdot 6H_2O$. Compare with A. F. Charles and D. A. Scott, *J. Biol. Chem.*, **102**, 425, 431, 437 (1933).

The following is a schematic representation of the essential postulates of Howell's theory:



The Nature of Prothrombin. Prothrombin occurs in association with the plasma globulins and has been prepared in relatively pure form by Mellanby,[28] Eagle,[29] and others. From the fact that it is acted on by trypsin and on the basis of other evidence it is probable that prothrombin is a protein.

The Nature of the Substance Derived from Platelets and Tissues. There is some uncertainty that this is identical with cephalin. Cephalin is

[28] *Proc. Roy Soc.* (*London*), *B*, **107**, 271 (1930).

According to Mellanby, *Proc. Roy. Soc.* (*London*), *B*, **113**, 93 (1933), thrombin or *thrombase* is a protein, soluble in water and destroyed by heat at temperatures above 50° C.; 1 mg. of thrombase coagulates 100 cc. of oxalated plasma in 30 seconds. The intravenous injection of 2 mg. into a rabbit causes intravascular clotting and death. Compare with A. Fischer, *Biochem. Z.*, **264**, 169, 178, 184 (1933).

[29] *J. Gen. Physiol.*, **18**, 531 (1935); see also *ibid.*, **18**, 547, 813 (1935). For a review of the subject, but more particularly for a summary of the basis for his theory, the reader is referred to Eagle's "Recent Advances in the Blood Coagulation Problem," *Medicine*, **16**, 95 (1937). Perhaps the most comprehensive review of the literature is that of E. Wöhlisch, "Die Physiologie und Pathologie der Blutgerinnung," *Ergeb. Physiol.*, **28**, 443–624 (1929). The subject has also been reviewed recently by Howell, "Theories of Blood Coagulation," *Physiol. Rev.*, **15**, 435 (1935).

much less active than crude tissue extracts or platelets.[30] Eagle [29] has suggested that the active coagulant is either some as yet unidentified substance, which is removed by lipoid solvents only in part, or that it is some complex molecule, such as lipoprotein, which is more active than its lipoid component. Eagle has submitted evidence in support of the view that the platelet (tissue) derivative and calcium together constitute a proteolytic enzyme which reacts with prothrombin to form thrombin. It is to be recalled that many years ago Morawitz postulated the theory that the platelet factor was an enzyme (*thrombokinase*) which promotes the formation of thrombin.

Thrombin itself is also considered to be a proteolytic enzyme, capable of acting on fibrinogen to form an insoluble split product, fibrin (Mellanby; [28] Eagle [29]). In addition to the direct evidence presented that the thrombin-fibrinogen reaction has the characteristics of an enzymatic reaction is the observation that papain, a proteolytic enzyme derived from the papaw plant, actively coagulates fibrinogen, forming soft clots resembling those produced by thrombin. Trypsin (including the pure crystalline enzyme) does not exert this effect, although it acts on prothrombin to form thrombin in a manner similar to that of the platelet derivative-calcium complex. Finally it is to be noted that certain snake venoms containing proteolytic enzymes coagulate fibrinogen independently of the presence of calcium, platelets, and prothrombin.

Eagle's theory of the mechanism of normal blood coagulation may be summarized as follows: [29,31,32]

$$\text{Prothrombin} + \underbrace{\text{calcium} + \text{platelets}}_{\text{Activating enzyme}} \rightarrow \underbrace{\text{thrombin}}_{\text{Enzyme product}}$$

$$\text{Fibrinogen} + \text{thrombin} \rightarrow \text{fibrin}$$

[30] Chargaff, Bancroft and Stanley-Brown have shown, however, that cephalin derived from yeast and soybean exerts a marked activating effect on plasma clotting Activity may be dependent on the degree of unsaturation, for synthetically prepared distearyl cephalin was found to be inactive. From defatted blood platelets a substance was extracted which exerted an inhibiting effect on blood clotting (*J. Biol. Chem.*, **116**, 237 [1936]).

[31] The activation of chymotrypsinogen by trypsin, resulting in the formation of chymotrypsin, is regarded by Eagle as a complete analogy to this hypothesis (Kunitz and Northrop, *J. Gen. Physiol.*, **18**, 433 [1935]).

[32] Other theories of coagulation may be briefly summarized. The theory of Schmidt (1872) is not only of historical interest, but may be looked upon as the classical theory, having served as the basis of the more orthodox of the later hypotheses. Schmidt believed thrombin to be formed from prothrombin and a zymoplastic substance and that it reacted with fibrinogen and a "fibrinoplastic substance" to form fibrin. Superficially Morawitz's theory (1904) seems to differ very little from present-day conceptions. He postulated the formation of thrombin through the reaction of thrombogen (plasma factor) with thrombokinase (from platelets) and with calcium. In the follow-up reaction thrombin and fibrinogen yielded fibrin. According to Bordet's theory, plasma contains fibrinogen and proserozyme, the latter being converted to serozyme by the action of calcium salts. The tissues, and particularly the platelets, contain a lipoprotein which Bordet named cytozyme and which he

The foregoing scheme does not include the factor or factors which maintain the normal fluidity of the blood. It is to be admitted that this aspect of the coagulation problem is at present unsolved, and though heparin may prove of significance in the prevention of intravascular clotting, its rôle is probably different from that indicated in the scheme on p. 228. According to Eagle, the quantities of heparin found in blood are only minute fractions of the amount required to prevent coagulation *in vitro*.

A second factor of possible importance is platelet stability. It is conceivable that, when blood is shed, the stability of the platelets is so altered that the active coagulating principle is released; this substance according to Eagle may well be a proteolytic enzyme resembling trypsin. Mention may be made in this connection of the occurrence in blood platelets, not only of a substance which accelerates coagulation, but of one which inhibits it.[30] Finally, it is to be noted that cysteine has been found to exert an inhibiting effect on blood coagulation (Mueller and Sturgis[33]).

Anticoagulants. The coagulability of the blood may be experimentally inhibited by the injection of sodium or potassium oxalate or citrate, peptone, hirudin, heparin, and other substances. With the exception of peptone, which acts only *in vivo*, the substances named are effective both *in vivo* and *in vitro*.

Oxalate is commonly used as an anticoagulant in obtaining blood for analysis. The effect is due to the precipitation of calcium, which is essential in the clotting process, as insoluble calcium oxalate.

Sodium citrate is chiefly used as an anticoagulant in blood transfusions. Its effect is due to the conversion of ionizable calcium salts into the much less ionized calcium citrate or calcium sodium citrate. Fluoride also prevents or delays clotting by combining with the calcium. Evidently, only the calcium ions are effective in the transformation of prothrombin into thrombin.

Heparin is effective in very small amounts, 1 mg. added to 25–50 cc. of blood being sufficient to delay coagulation for as long as 24 hours.

Hirudin is the active principle derived from the salivary gland of the medicinal leech. It is said to be a proteose, preventing coagulation by neutralizing thrombin.

The injection of toxic doses of proteose or peptone renders the blood incoagulable, presumably through a depletion of fibrinogen. Whether

believed to be lecithin. Cytozyme, according to the theory, unites with serozyme to yield thrombin, which in turn transforms fibrinogen into fibrin. Mills has advanced the view that there are two distinct mechanisms concerned with the coagulation of blood. In addition to the thrombin mechanism, he believes that clotting may be produced by the action of tissue fibrinogen on plasma fibrinogen, even in the absence of thrombin or prothrombin. These and other theories are discussed and criticized in the reviews by Wöhlisch, Howell, and Eagle.[29]

[33] *Science*, **75**, 140 (1932).

this is the only factor is not clearly established. Peptone is said to increase the amount of antithrombin.

At low temperatures blood tends to remain fluid for a long time. This has been associated with the greater resistance of platelets in the cold and the consequent delay in the liberation of cephalin. On the contrary, heat favors the clotting of blood.

Clotting may also be delayed by collecting blood in a paraffin-coated vessel (non-wettable surface). This condition is also believed to diminish the fragility of the platelets.

Coagulation Time, Hemophilia, Thrombocytopenia, etc. In man the clotting time of blood is normally 2–10 minutes, varying somewhat according to the method used for its determination.

The most striking departure from the normal occurs in *hemophilia.* This disease is manifested clinically by a tendency to excessive bleeding and prolonged clotting time. Hemorrhage may be spontaneous, or may follow an injury, even of the most trivial kind. The condition is hereditary, the transmission being sex-linked, manifested only in males, but transmitted through females.

The blood of an hemophiliac frequently requires 40–50 minutes, and occasionally even more than two hours, for clotting. And yet, it apparently contains all the necessary elements for the coagulation process (prothrombin, calcium, platelets, and fibrinogen).

It has been suggested that, although the number of platelets is normal in hemophilia, they are abnormally resistant to disintegration (Howell and Cekada [34]) and in consequence the cephalin does not become available for the clotting process as readily as in normal blood. Evidence has been submitted that platelet suspensions of normal and hemophilic bloods are essentially equal in their ability to hasten the coagulation of normal blood, but only normal platelets shorten the clotting time of hemophilic blood. This is not supported by the work of recent investigators. Patek and Stetson [35] found that, in the clotting of either hemophilic or normal plasma, hemophilic platelets behave like normal platelets. The defect, they believe, is not in the platelets but in the plasma.

It has been determined that there is no *quantitative* difference in the amount of prothrombin in normal and hemophilic plasma, and indeed, when the globulins derived from these two sources are tested with a calcium-fibrinogen system, the same degree of activity is exhibited. This has been confirmed by Patek and Taylor,[36] whose work has also disclosed that the globulin fraction of normal, but not of hemo-

[34] *Am. J. Physiol.*, **78**, 500 (1926).

[35] *J. Clin. Investigation*, **15**, 531 (1936); compare with G. R. Minot and R. I. Lee, *Arch. Internal Med.*, **18**, 474 (1916).

[36] *J. Clin. Investigation*, **16**, 113 (1937); see also H. Eagle, *J. Gen. Physiol.*, **18**, 813 (1935), and A. J. Quick, M. Stanley-Brown, and F. W. Bancroft, *Am. J. Med. Sci.*, **190**, 501 (1935).

philic, plasma contains a substance capable of accelerating clotting of hemophilic blood. From this it may be inferred that the coagulative deficiency in hemophilia is due to a *qualitative* difference of the prothrombin, a theory that is not entirely new, as it was partly realized by Addis [37] in 1911.

The use of ovarian hormone preparations in the treatment of hemophilia was recommended by Birch [38] on the supposition that, albeit females potentially have the disease, it is held in abeyance by an internal secretion from the ovary. However, other clinicians [39] have been unable to confirm the therapeutic efficacy of ovarian hormone in hemophilia.

Of the various hemorrhagic diseases classified under the head of "*purpura*," the relation to blood clotting is understood only in the so-called *purpura hemorrhagica*, or *thrombocytopenic purpura*. In this condition the blood ordinarily has a *normal clotting time*, although the clot is usually soft and fails to retract, but the bleeding time is greatly prolonged. Normally after a slight puncture there is rapid diminution in the intensity of the hemorrhage, successive drops of the blood being smaller and smaller, and soon the bleeding stops altogether. Cessation of the bleeding is brought about by the formation of small thrombi in the injured capillaries, the thrombi being made up essentially of aggregations of blood platelets. In the hemorrhagic diseases referred to, there is no prompt checking of the hemorrhage, which may continue for hours, the drops showing little or no tendency to diminish in size. This prolongation in the bleeding time is attributed to a deficiency of platelets, which in severe hemorrhagic diseases have often been found to be as low as 10,000 per cubic millimeter, or even less.

Not all are agreed that platelet deficiency is the principal cause of thrombocytopenic purpura, but that other factors, such as increased capillary permeability and inability of the blood vessels to contract, may be of primary importance.

In conditions of severe liver damage, such as occur in chloroform and phosphorus poisoning and in acute yellow atrophy, there is deficient formation of fibrinogen, as a result of which its content in the blood may fall to very low values. To this reduction of fibrinogen is attributed the delayed clotting time and hemorrhagic tendency in these conditions.

As has been mentioned, the removal of calcium from the blood as the oxalate or citrate prevents its clotting. However, physiologically, diminished clotting of the blood is not frequently attributable to a diminished calcium content of the blood. It is perhaps a factor in obstructive

[37] *J. Path. Bact.*, **15**, 427 (1911).

[38] C. L. Birch, *J. Am. Med. Assoc.*, **99**, 1566 (1932); *Proc. Soc. Exptl. Biol. Med.*, **28**, 752 (1931). H. T. Kimm and C. M. van Allen, *J. Am. Med. Assoc.*, **99**, 991 (1932).

[39] R. P. Stetson, C. E. Forkner, W. B. Chew, and M. L. Rich, *J. Am. Med. Assoc.*, **102**, 1122 (1934); Chew, Stetson, G. Van S. Smith, and O. W. Smith, *Arch. Internal Med.*, **55**, 431 (1935); H. W. Jones and L. M. Tocantins, *J. Am. Med. Assoc.*, **103**, 1671 (1934).

jaundice, for the "coagulability" of the blood of patients with this disease may be increased by the administration of calcium salts. There is some evidence, however, that the hemorrhagic tendency in jaundice is brought about by a diminution of prothrombin (Quick [40]).

Intravascular Clotting. Certain circumstances favor the clotting of blood in the blood vessels, clots thus formed being called *thrombi*, and the process *thrombosis*. Intravascular clotting may result from the slowing down of the blood flow, such as may occur in heart disease, or where there is an abnormal dilation of the blood vessels, as in varicose veins, or where there is some obstruction in the vessel. A thrombus also tends to form around an area of injury, such as an atheromatous patch in an artery, a sclerosed coronary vessel, or on a damaged heart valve. Infections, such as typhoid fever, predispose to thrombus formation, and there are other causes which are described in textbooks of pathology.

The formation of intravascular clots differs from clotting outside the blood vessels. At first, there is a clumping together of blood platelets, forming a framework, which becomes infiltrated by white blood cells. At this stage, the thrombus is whitish in color and is called a white thrombus. When its size is sufficient, it causes obstruction of the blood vessel, the flow of blood is stopped, and a red blood clot, the red thrombus, is formed. This consists of fibrin and all the blood elements. It fills the obstructed blood vessel to the point of its nearest anastomosis with some other vessel.

Globulin and Albumin. The separation of serum protein into two fractions may be accomplished by half-saturation with ammonium sulfate. The globulin is salted out while the albumin remains in solution. Separation of these two fractions may also be performed in 1.5 molar sodium sulfate (approximately 22 per cent). In turn the globulin may be divided into two fractions, *euglobulin* and *pseudoglobulin*, the former being precipitated in 1.0 molar sodium sulfate.[41] Although this classification is quite generally employed, the chemical individuality of the fractions is far from established and the further subdivision of the albumin and globulin has been questioned even more from the standpoint of the chemical identity of the various subfractions.

The question of the individuality of the serum protein fractions may be studied from different angles, but in this connection only a brief statement is possible. The peculiarities of serum protein behavior and the complexity of the problem may be illustrated by Sørensen's observation that many-times-recrystallized serum albumin can be fractionated into a variety of crystalline albumins, of differing physical and chemical properties. In turn these may be recombined and, if this is done in the correct proportions, the original albumin is reconstructed. Upon these and similar results is based Sørensen's theory of the structure of proteins (p. 101).

[40] *Am. J. Med. Sci.*, **190**, 501 (1935).
[41] P. E. Howe, *Physiol. Rev.*, **5**, 439 (1925).

A plausible analysis of the problem has been made by Block,[42] whose opinion concerning the question of whether serum contains one or more than one completely independent protein fraction is that it cannot be definitely answered. He postulates the existence of two principal coprecipitation systems which are interdependent, yet possess a sufficient degree of independence to permit their separation one from another. The two major fractions are albumin and globulin, which may be further fractionated to yield the various crystalline and amorphous proteins which have been found in serum. Block has coined the term *orosin* (Gr. meaning serum) to designate the total coagulable protein of serum.

McFarlane,[43] in a remarkable study of ultracentrifugal sedimentation of serum protein, has shown that the separation into fractions by this method yields quantitatively different results from those obtained by precipitation. In dilute mixtures the heavier fraction has, however, the characteristics of salted-out globulin and the lighter fraction corresponds to serum albumin, but in concentrated mixtures the proteins are not so sharply distributed and, hence, are not similarly identifiable. Another significant observation is that when serum is diluted the fall in colloid osmotic pressure is correspondingly greater than the fall in protein concentration, indicating an increase in the average molecular size of the protein on dilution. By simply diluting serum the ratio of the fractions obtained by ultracentrifugal analysis approaches the analytical value for the albumin-globulin ratio.[44] Ultracentrifugal sedimentation yields higher results for albumin and lower results for globulin than are obtained by chemical analysis. This may mean that in the physicochemical treatment of the serum such as salting-out preliminary to its analysis, the proteins may have been altered, as regards their molecular size and possibly in other respects as well, and that therefore the fractions into which the proteins have been separated have no real being in the serum itself.

From the foregoing it is seen that the terminology of albumin and globulin is merely a matter of convenience, and, as others have pointed out, the available data are insufficient to define accurately the true nature of either molecular fraction. However, most workers who have attempted to determine the molecular weight of serum albumin have regarded it as an entity. Thus Svedberg and Sjogren have stated that once-crystallized serum albumin is a homogeneous substance of molecular weight 68,000 (67,100, according to more recent calculation, p. 106). In their hands repeated crystallization apparently resulted in the decomposition of the protein. This was not observed by Adair and Robinson,[45]

[42] *Yale J. Biol. Med.*, **9**, 445 (1937).

[43] *Biochem. J.*, **29**, 407, 660, 1175, 1209 (1935).

[44] McFarlane has also submitted evidence for the presence in serum (horse, cow, man) of three molecular types of protein, albumin, globulin, and a third fraction designated *X*.

[45] *Biochem. J.*, **24**, 1864 (1930).

who found serum albumin to remain stable through several crystallizations. Moreover, they could detect no change in the osmotic pressure of serum albumin preparations, crystallized once, twice, and four times. The mean molecular weight obtained in a series of 27 determinations with horse-serum albumin was 72,000 ± 3000. Serum albumin of the ox and sheep yielded approximately the same result (molecular weight about 70,000).

For unfractionated serum globulin Adair and Robinson obtained a mean molecular weight of 175,000, the range of values in 17 experiments being from 154,000 to 192,000. Euglobulin resembled total globulin. These investigators could find no explanation for the discrepancy between their result and Svedberg's value for total globulin of only 103,000; however, more recently (p. 106) Svedberg has given 150,000 as the approximate molecular weight of serum globulin (horse).

Normal Distribution of Albumin and Globulin. Moore and Van Slyke [46] observed the following range of values in nine normal subjects:

TABLE XXXII

	Total Plasma Protein, per cent	Albumin, per cent	Globulin, per cent	$\frac{\text{Albumin}}{\text{Globulin}}$
Minimum	6 5	4 0		
Maximum	7 7	4 5		
Average	7 1	4 3	2 8	1.53

The amount of albumin normally exceeds the globulin; the proportion though variable is usually from 1.5 : 1 to 2.0 : 1.

Functions of the Plasma Proteins. One of the important functions of the plasma proteins is to maintain the normal osmotic relations between the blood and tissues. Starling [47] was the first to recognize the significance of the protein of the blood as a controlling factor in the distribution of fluids between the plasma and tissues.

The osmotic pressure of plasma is about 6.5 atmospheres (494 cm. Hg). This tremendous force, which is due to dissolved electrolytes and organic crystalloids, does not produce, however, the calculated effect, because it is *almost* wholly balanced by the osmotic pressure of the tissue fluids, which also contain these substances in about the same concentration. Such excess in osmotic pressure as the blood possesses, even though it represents only a small fraction of the total, is nevertheless of incalculable importance. It corresponds to the difference in concentration of protein in the plasma and tissue fluids, a difference

[46] *J. Clin. Investigation*, **8**, 337 (1929–30); see also A. E. Kumpf, *Arch. Pathol.*, **11**, 335 (1931).

[47] *J. Physiol.*, **19**, 312 (1895–6).

that is maintained because of the relative impermeability of the capillary endothelium to protein.

Krogh [48] states that in his laboratory determinations of the osmotic pressure of the serum in 12 normal subjects gave an average result of 380 mm. of water (equivalent to 27.94 mm. Hg). Together with other data in the literature based on measurements with various types of osmometers, the normal variations are given as 265 to 420 mm. of water pressure. In a series of determinations on 11 normal subjects (medical students), Fellows [49] obtained values ranging from 321 to 380 mm. The total serum proteins in these subjects varied from 6.32 to 6.81 grams.[50]

For the purpose of the present discussion the osmotic pressure of the blood (due to the plasma protein) may be assumed to be approximately 28 mm. Hg. As protein is also present in tissue fluid, the osmotic effect which it exerts cannot be disregarded in considering the forces which regulate the exchange of fluid between the blood and tissues. Certain fluids, such as cerebrospinal and intraocular, contain a very small amount of protein (p. 294); hence their colloid osmotic pressure may be ignored, but under certain conditions the protein concentration of these fluids, and even normally of tissue fluids elsewhere, may become so high as to exert a significant osmotic effect. In all probability this rarely exceeds 10 mm. Hg. Accordingly the *effective* osmotic pressure of the blood is normally about 18 mm. Hg (28–10).

Although retention of fluid in the blood vessels depends on the protein content of the plasma and more specifically on the difference in osmotic pressure between plasma and tissue fluid, the outward filtration of water and contained solutes is promoted by the hydrostatic pressure in the capillaries. This probably varies considerably in different locations of the body; in the capillaries of the skin it is about 32 mm. Hg (Landis [51]), a value which in this discussion may be accepted as the average pressure in the arteriolar portion of the capillaries generally. This head of pressure falls off gradually to about 12 mm. in

[48] "The Anatomy and Physiology of Capillaries," Yale Univ. Press, New Haven, 1929, pp. 286–290.

[49] *Proc. Soc. Exptl. Biol. Med.*, **29**, 1175 (1931–32).

[50] The validity of determinations by the osmometer method has been recently discussed by Turner (*J. Biol. Chem.*, **96**, 487 [1932]), by Wells, Miller, and Drake (*J. Clin. Investigation*, **14**, 1 [1935]), and others. An automatic recording osmometer has been devised by Sumwalt and Landis (*J. Lab. Clin Med.*, **22**, 402 [1937]), and Yanagi has constructed an osmometer which permits periodic observation of pressure and insures sterility of the serum (*J. Clin. Investigation*, **14**, 853 [1935]). See also Wies and Peters, *J. Clin. Investigation*, **16**, 93 (1937), and Keys and Taylor, *J. Biol. Chem.*, **109**, 47 (1935).

[51] *Am. J. Physiol.*, **75**, 548 (1926). Landis determined the pressure of the capillaries of the frog's mesentery to be 14.5 cm. (H_2O) in the arterial end and 10 cm. in the venous. Filtration outward occurred when the pressure was above 11.5 cm., which is to be expected from the relatively low plasma protein osmotic pressure of the frog (10 to 12 cm., according to White, *Am. J. Physiol.*, **68**, 523 [1924]).

the venous limb of the capillaries, and in the lymphatics the pressure is still less.

From this it is seen that at the arterial end the hydrostatic pressure exceeds the colloid osmotic pressure (32 − 18 = + 14), a condition that favors filtration, or transudation of water and salts outward through the capillary wall. At the venous end the condition is reversed; here the osmotic pressure, being essentially the same as in the arterioles, is therefore greater than the hydrostatic pressure (12 − 18 = − 6), a circumstance that favors reabsorption of fluid into the blood vessels by filtration inward through the capillary wall. In short the gradient of capillary pressure is balanced against the colloid osmotic pressure in such a way that under average conditions the filtration occurring in the arteriolar portion of the capillary network is balanced by absorption in the venous capillaries and small venules (Landis).

What happens when the venous pressure is abnormally increased has been shown by Landis [52] in an experiment in which the venous pressure in one arm was raised by means of an armlet to 20, 40, 60, or 80 mm. Hg and the composition of the blood in that arm compared with that of blood collected from the control arm, where the pressure had remained at its normal value of 9 mm. Loss of fluid could be detected at venous pressures as low as 20 mm. The loss was conspicuously increased at higher venous pressures, as much as 19.5 per cent passing out into the tissues when the venous pressure was raised to 80 mm. The amount of protein lost was negligible below 60 mm. At that point the capillary filtrate contained only 0.3 per cent protein; at 80 mm., however, the filtrate contained an average of 1.5 per cent protein.

It is thus seen that increased hydrostatic pressure in the capillaries may be accompanied by a loss of fluid to the tissues, and up to a certain point by an increased concentration of protein in the plasma because of a proportionately smaller loss of this constituent. The greater concentration of protein tends to balance the effect of the heightened hydrostatic pressure, so that within certain limits a new balance is soon attained.

Calculation of Osmotic Pressure of Serum Proteins from Analytical Data. Govaerts [53] determined that for each gram of albumin per 100 cc. of serum there was an osmotic pressure of 75.4 mm. of water (5.54 mm. Hg), whereas 1 gram per cent of globulin exerted a pressure of 19.5 mm. of water (1.43 mm. Hg). According to these figures, the osmotic pressure of serum albumin is nearly four times that of globulin. Using these constants as a basis for calculation, Govaerts was able to predict the osmometric reading from the chemical analysis of the serum for albumin and globulin. Govaerts' formula may be written as follows:

$$P = A \times 5.54 + G \times 1.43$$

[52] E. M. Landis, L. Jonas, M. Angevine, and W. Erb., *J. Clin. Investigation*, **11**, 17 (1932).

[53] *Compt. rend. soc. biol.*, **93**, 441 (1925); **95**, 724 (1926).

where P is the osmotic pressure in millimeters of mercury, A the grams of albumin, and G the grams of globulin in 100 cc. of serum (or plasma).

In a similar comparison made by Fellows,[49] Govaerts' constants yielded somewhat higher results than those obtained by osmometry. Wells and associates[54] consider the formula of Govaerts unreliable. They propose the following formula:

$$P = C(21.4 + 5.9A)$$

where P is the osmotic pressure in millimeters of water, C is the total protein concentration, and A is the albumin concentration in grams per 100 cc.

Wies and Peters[55] have recently reëxamined the relation of the concentration of serum protein, and its fractions, albumin and globulin. They derived the following empirical equation for the estimation of colloid osmotic pressure:

$$\pi = 60.9 \times A_w + 22.9 \times G_w - 50$$

where π is the osmotic pressure as millimeters of water; A_w the grams of albumin, and G_w the grams of globulin, per kilo of water.[56]

Protein Depletion and Regeneration. Serum protein may be reduced to a very low level by repeated bleeding and simultaneous injection of the washed blood cells suspended in a protein-free medium, such as Locke's solution. This procedure, termed *plasmapharesis*, was employed by Kerr, Hurwitz, and Whipple[57] in a study of serum protein regeneration. It will be recalled that the regeneration of fibrinogen is very rapid. Not so with the other proteins. Following a 50 per cent depletion, it required, after an initial rise of about 1 per cent during the first 24 hours, 7 to 14 days to restore the lost protein. Regeneration was more rapid on a meat diet than on a protein-free diet. It was somewhat retarded in the presence of liver injury. Globulin was regenerated more rapidly than albumin. Whipple and associates found that the body cannot tolerate too great a reduction of its plasma protein. Depletion to a concentration of 1 per cent was almost always accompanied by fatal shock; this often supervened long before the minimum figure of 1 per cent was reached.

Plasmapharesis as a method of producing experimental edema was first developed by Leiter,[58] who found that in dogs palpable edema usu-

[54] H. S. Wells, J. B. Youmans and D. G. Miller, *J. Clin. Investigation*, **12**, 1103 (1933).

[55] *J. Clin. Investigation*, **16**, 93 (1937).

[56] The concentrations of serum protein and its fractions may be corrected to grams of protein per kilo of water by means of the equation $W = 98.4 - 0.718\,P$. See A. J. Eisenman, L. B. Mackenzie, and J. P. Peters, *J. Biol. Chem.*, **116**, 33 (1936).

[57] *Am. J. Physiol.*, **47**, 356, 370, 379 (1918–19); see also H. P. Smith, A. E. Belt, and G. H. Whipple, *ibid.*, **52**, 54 (1920).

[58] *Proc. Soc. Exptl. Biol. Med.*, **26**, 173 (1928); *Arch. Internal Med.*, **48**, 1 (1931).

ally begins when the plasma protein has fallen to 3 per cent, or less, and recedes with a rise above this critical level. The edema fluid was characterized by its low protein content. The mechanism of the development of this form of edema may be adequately explained on the basis of the lowered osmotic pressure of the plasma proteins; however, the degree of fluid accumulation in the tissues and the rate are modified by the intake of water and salt, the chief constituents of the edema fluid.

Edema. We are not concerned at this stage with the types of edema resulting primarily from heart disease, venous or lymphatic obstruction, acute glomerular nephritis, or other causes, but with the forms of fluid accumulation in the tissues in which low plasma protein is the dominant factor. Accumulation of fluid in the tissues in man usually becomes evident when the level of serum protein falls below 5 grams per 100 cc. This so-called " edema level " is subject, however, to variation, depending on the relative proportions of albumin and globulin.

According to the observations of Iversen and Nakazawa,[59] edema develops when the colloid osmotic pressure of the serum (due to proteins) falls below 250 mm. H_2O.

Rôle of the Liver. Whipple's observation of retarded protein regeneration in the presence of liver injury has been mentioned. The more recent experimental studies of Luck,[60] Addis,[61] and others have made it clear that the liver contains a labile reserve of protein that is readily depleted during fasting and restored when protein is fed, the rate and degree of enrichment being greater on a high-protein diet than on a low one.

Hypoproteinemia occurs in cirrhosis of the liver, as well as in other forms of hepatic disease. The reduction in plasma protein is often sufficient to result in edema. In certain instances the protein deficiency may be explained on the basis of an inadequate protein intake; where considerable ascites occurs, a certain amount of plasma protein is lost to the ascitic fluid; but the most important factor, judging from the work of Myers and Keefer,[62] is probably an alteration in liver function and defective formation of plasma protein.

Thompson, McQuarrie, and Bell [63] have reported the case of a child in whom edema was associated with hypogenesis of serum protein and atrophic changes in the liver. The latter was demonstrated *post mortem*, while the relationship of the low serum protein to the edema was shown by the fact that an elevation of the plasma protein concentration by means of blood transfusion resulted in the complete, though transitory, disappearance of the edema.

[59] *Biochem. Z.*, **191**, 307 (1927).
[60] *J. Biol. Chem.*, **115**, 491 (1936).
[61] T. Addis, L. J. Poo, and W. Lew, *ibid.*, **116**, 343 (1936).
[62] *Arch. Internal Med.*, **55**, 349 (1935).
[63] *J. Pediatrics*, **9**, 604 (1936).

Edema Due to Malnutrition. Chronic malnutrition, from whatever cause, and particularly dietary deficiency of protein, is invariably associated with a decrease in plasma protein. The albumin fraction is especially affected because it is formed with greater difficulty than globulin. If the reduction in plasma protein, and especially of albumin, is sufficient, edema develops.

This correlation has been established quite recently. During and after the World War, nutritional edema, commonly called " war edema," was prevalent in famine areas and in prison camps.[64] Authorities at first attributed it to various causes, but there is now little doubt that the underlying factor was protein deficiency. Moreover, nutritional edema has been produced experimentally in rats and dogs maintained on low-protein diets.[65]

Youmans [66] has reported the occurrence of edema in clinic patients whose usual diets were low, or minimal, in total calories and in protein. Although the total plasma protein was often within the limits of normal, the albumin was usually reduced, while the globulin was increased. The occurrence of chronic low-grade deficiency in serum protein is common among poor children, subsisting on protein-deficiency diets. Such children are predisposed to the development of edema, especially in the presence of infection (Dodd, Minot, and Keller [67]).

Edema Associated with Nephrosis. Nephrosis is a chronic disease in which two of the chief manifestations are the excretion of large quantities of protein in the urine and edema. There has accumulated a large body of evidence that it is due to the lowered osmotic pressure of the plasma. The protein fraction excreted by the kidney in greater proportion is albumin, which possesses the greater osmotic effect. It is also the protein that is less easily regenerated. In consequence, the albumin-globulin ratio tends to become very low, values of 0.3 being observed not infrequently. The osmotic pressure is correspondingly lowered. Krogh has recorded a colloid osmotic pressure in one case of only 100 mm. of water, as compared with a capillary blood pressure of 150 mm. There was therefore in this case a head pressure of 50 mm. in favor of filtration from the blood into the tissue spaces.[68]

[64] The historical aspects of nutritional edema have been reviewed by M. B. Maver, *J. Am. Med. Assoc.*, **74**, 934 (1920).

[65] E. A. Kohman, *Am. J. Physiol.*, **51**, 378 (1920); R. A. Frisch, L. B. Mendel, and J. P. Peters, *J. Biol. Chem.*, **84**, 167 (1929); see also Shelburne and Egloff, *Arch. Internal Med.*, **48**, 51 (1931); Weech, Snelling, and Goettsch, *J. Clin. Investigation*, **12**, 193 (1933).

[66] *J. Am. Med. Assoc.*, **99**, 883 (1932); *Arch. Internal Med.*, **50**, 843 (1932); **51**, 45 (1933).

[67] *J. Pediatrics*, **8**, 442, 452 (1936).

[68] For a review of the literature on nephrosis, the reader is referred to L. Leiter, *Medicine*, **10**, 135 (1931).

The nature of serum (and urine) proteins in nephrosis has been the subject of a number of recent investigations. See E. M. Widdowson, *Biochem. J.*, **27**, 1321

The edema fluid in nephrosis is characteristically low in its protein content, just as in the edemas resulting from malnutrition or plasmapharesis. Many respond favorably to a high-calorie and moderate to high meat intake.

Other Physiological and Pathological Variations. A reduction in plasma protein occurs during pregnancy, this being partly attributable to blood dilution and partly to protein depletion, the latter because of the fetal requirements, restricted protein intake, and possibly other factors. The albumin-globulin ratio is diminished and the fibrinogen increased.

The plasma protein concentration is low in infancy, but attains adult values within the first two years of life.

Dehydration resulting from water deprivation, muscular activity, diarrhea, vomiting, and other causes is associated with a diminished plasma volume and an increased protein concentration.

In acute hemorrhagic nephritis (acute glomerular nephritis), there seems to be a generalized increase in capillary permeability, presumably due to some toxic factor. There is an escape of protein not only through the glomeruli, but also through the capillaries into the tissues generally, so that the edema fluid in this condition tends to be relatively high in protein (1 per cent, or more). The development of edema is therefore not related primarily to protein deficit, though it is obvious that, with the loss of protein from the blood, its content is thereby diminished.

Plasma protein frequently remains unchanged in so-called arteriosclerotic Bright's disease. The low plasma protein often seen in patients with heart failure usually has a nutritional basis.

Hyperproteinemia is encountered in multiple myeloma, in which disease 12 grams or more of protein per 100 cc. of plasma is not a rare finding.

HEMOGLOBIN

Hemoglobin, the pigment of the red blood corpuscles, belongs to the group of conjugated proteins. One of its most characteristic properties is that of combining with oxygen to form oxyhemoglobin, a compound which is readily dissociated when exposed to an environment of low oxygen tension. The iron content of hemoglobin is practically the same for most species of animals and amounts to about 0.335 per cent. On the assumption that the hemoglobin molecule contains at least one atom of Fe, the minimum molecular weight has been calculated to be about 17,000. However, it is very probable that the hemoglobin molecule contains not one but four atoms of iron. The osmotic pressure determinations of Adair [69] have led to the value of 68,000, or four times the minimal molecular weight. Confirmation of this value has been

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(1933); E. Goettsch and E. B. Reeves, *J. Clin. Investigation*, **15**, 173 (1936); A. S. Alving and A. E. Mirsky, *ibid.*, **15**, 215 (1936).

[69] *Proc. Roy. Soc.* (*London*), *A*, **109**, 292 (1925).

obtained by several investigators, employing different experimental methods.[70]

Crystallization of Hemoglobin. Hemoglobin may be crystallized with relative ease from the blood of certain animals, such as the horse, dog, and guinea pig. The hemoglobins of the ox and rat crystallize with

FIG 22.—Oxyhemoglobin of the White Rat.

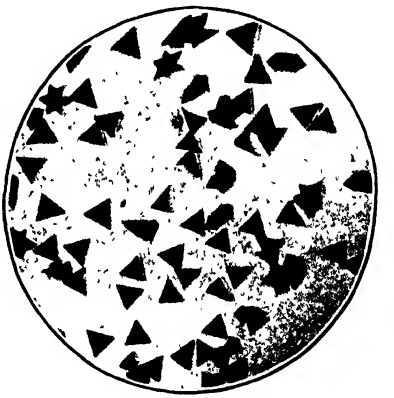


FIG. 23.—Oxyhemoglobin of the Guinea Pig.

FIG. 24.—Oxyhemoglobin of Man. (After Otto Funke, "Atlas of Physiological Chemistry." (Printed for the Cavendish Society, London [1853], Plate X.)

difficulty, whereas other hemoglobins, such as that of the frog, have not been obtained in crystalline form. The most important study of the crystalline structure of the hemoglobins is that of Reichert and Brown,[71]

[70] Svedberg and Fahraeus, *J. Am. Chem. Soc.*, **48**, 430 (1926); Svedberg and Nichols, *ibid.*, **49**, 2920 (1927); Vickery and Leavenworth, *J. Biol. Chem.*, **79**, 377 (1928); Northrop and Anson, *J. Gen. Physiol.*, **12**, 543 (1929).

[71] "The Crystallography of Hemoglobins," Carnegie Institution of Washington, 1909.

who showed that oxyhemoglobin crystals differ not only with the genus but also with the species of the animal from which they are obtained, no two hemoglobins forming identical crystals. There is, however, similarity in the crystal structure of the hemoglobins of genetically related animals, as for example the horse and donkey. Indeed the hemoglobin of these animals cannot be differentiated immunologically.[72] Since heme, the non-protein part of all hemoglobins, is the same, the specificity and possible differences in the constitution of different hemoglobins presumably reside in the globin. When Reichert and Brown were engaged in their monumental work, the importance of the reaction and salt concentration was not fully appreciated. As has been pointed out by Hastings,[73] it would be of considerable importance if a crystallographic study comparable to that of Reichert and Brown were made today on crystals from isoelectric, salt-free solutions of hemoglobin.

Absorption Spectra. When white light is transmitted through solutions of hemoglobin, or of compounds related to it, certain wavelengths are absorbed, with the result that these solutions, when examined spectroscopically, exhibit absorption spectra. Oxyhemoglobin or diluted arterial blood shows two absorption bands between the Fraunhofer lines D and E, one narrower than the other. The center of the narrower or α band corresponds to the wavelength $\lambda = 579$ mμ, and that of the second or β band to $\lambda = 542$ mμ. The β band disappears first on dilution. A third band, having its center at $\lambda = 415$ mμ, i.e., in the extreme violet region, may be seen in spectrophotographs of oxyhemoglobin.[74]

Hemoglobin (reduced hemoglobin) shows a spectrum with one broad band between D and E and nearer to D. The center and darkest part of the band corresponds to the wavelength $\lambda = 559$ mμ.

Methemoglobin is formed when blood is treated with ozone, potassium permanganate, potassium ferricyanide, chlorates, nitrites, nitrobenzene, pyrogallol, acetanilide, and many other substances. These compounds when introduced into the organism cause the appearance of methemoglobin both in the blood and urine. One hydrogen equivalent of oxidizing agent is presumably required in the conversion of reduced hemoglobin into methemoglobin. The Fe in methemoglobin is in the ferric state, Fe^{+++}, whereas in hemoglobin and oxyhemoglobin it is in the ferrous condition, Fe^{++}. In acid solution, methemoglobin shows one band, the center of which corresponds to a wavelength of about $\lambda = 634$ mμ.

[72] Boor and Hektoen in a study of the antigenic properties of carbon-monoxide hemoglobin (carefully purified by repeated crystallization) confirmed the species specificity of hemoglobin and its derivatives. They obtained cross reactions in closely related species, as duck, chicken, and turkey; beef and sheep. *J. Infectious Diseases*, **46**, 1 (1930).

[73] Colloid Symposium Monograph," **6**, 140 (1928).

[74] The amount of light transmitted may be accurately measured by modern spectrophotometric methods. See for example D. L. Drabkin and J. H. Austin, *J. Biol. Chem.*, **98**, 719 (1932); **112**, 51, 67, 89 (1935).

The carbon monoxide-hemoglobin spectrum shows two bands; the middle of the first corresponds to wavelength $\lambda = 570$ mμ, and that of the second to $\lambda = 542$ mμ. Carbon monoxide hemoglobin absorption spectra can be distinguished from oxyhemoglobin spectra by the fact that reducing substances, such as ammoniacal ferrous tartrate (Stokes' reagent), have a less marked effect on the absorption bands of carbon monoxide hemoglobin than on those of oxyhemoglobin.[75] Hydrogen sulfide hemoglobin and cyanhemoglobin (hydrocyanic acid hemoglobin) likewise give characteristic absorption spectra. They are formed in the blood of individuals poisoned with hydrogen sulfide and cyanide, respectively.

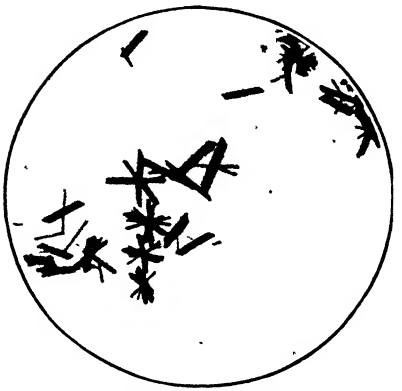


FIG. 25. — Hemin crystals. (After Nencki and Zaleski, *Z. physiol. Chem.* **30**, 423 [1900].)

Chemistry of Hemoglobin; Relation to Heme and the Porphyrins. When hemoglobin is treated, under appropriate conditions, with glacial acetic acid and sodium chloride, and the mixture warmed gently, a substance is obtained which crystallizes readily as brown crystals (Fig. 25). This substance is hemin, $C_{34}H_{32}N_4O_4FeCl$.

The other product of the cleavage is globin. Hemin has been synthesized by Fischer and Zeile.[76] It contains four methylpyrrole

[75] Carbon monoxide has a greater affinity for hemoglobin than oxygen. The relationship between the proportions of hemoglobin that would unite with oxygen and carbon monoxide at varying gas pressures has been formulated by Douglas, Haldane, and Haldane (*J. Physiol.*, **44**, 275 [1912]), and is expressed mathematically by the equation:

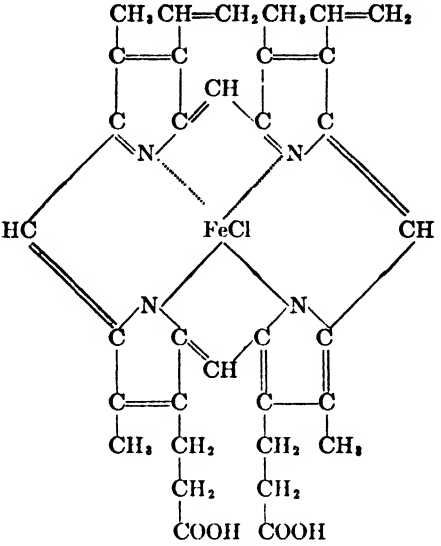
$$\frac{[HbCO]}{[HbO_2]} = K \frac{{}_pCO}{{}_pO_2}$$

The brackets indicate the concentrations of hemoglobin combined as carbon monoxide hemoglobin and as oxyhemoglobin; ${}_pCO$ and ${}_pO_2$ the gas tensions, and K the relative affinity constant for hemoglobin for the two gases.

A recently estimated value of K shows that the tendency of hemoglobin (of hemolyzed human blood) to form carbon monoxide hemoglobin is approximately 210 times greater than the tendency to form oxyhemoglobin (J. Sendroy, S. H. Liu, and D. D. Van Slyke, *Am. J. Physiol.*, **90**, 511 [1929]). When carbon monoxide is breathed, a large proportion of the hemoglobin combines with it. If thereby the amount left to combine with oxygen is sufficiently diminished, the tissues do not obtain sufficient oxygen to maintain life, and death from asphyxiation results. In non-fatal cases of severe carbon monoxide poisoning, permanent injury to the central nervous system may occur, often consisting of a softening of the lenticular nuclei, with a resulting syndrome of paralysis agitans.

[76] *Ann.*, **98**, 468 (1929). It is not definitely known to which pair of nitrogen atoms the iron atom is attached.

radicals and an atom of iron and may be represented by the following structural formula:

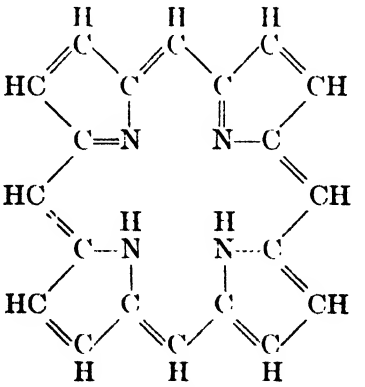


Hemin

If the crystals of hemin are treated with sodium hydroxide, the corresponding base is liberated. The reaction may be represented by the equation:

$$\underset{\text{Hemin}}{C_{34}H_{32}N_4O_4FeCl} + NaOH = \underset{\text{Heme}}{C_{34}H_{32}N_4O_4FeOH} + NaCl$$

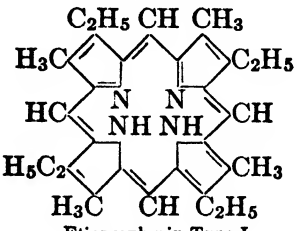
Heme is a derivative of one of a large and widely distributed group of compounds known as *porphyrins*. These are iron-free pigments containing the *porphin* nucleus, which is composed of four pyrrole groups united by four methene (=CH—) linkages.



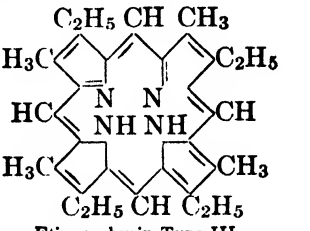
Porphin Nucleus

In the naturally occurring porphyrins the hydrogen atoms attached to the carbons are substituted by various groups and radicals. Similar compounds have been obtained as laboratory products. From the structure of the porphin nucleus it may be surmised that the number of possible porphyrins and their derivatives must be very large, and indeed it has been brought out that hemin, protoporphyrin, hematoporphyrin, mesoporphyrin, deuteroporphyrin and deuterohemin each have 15 possible isomers. Etioporphyrin (aetioporphyrin) has four possible isomers, all of which have been synthesized.[77]

Of the four etioporphyrins, two (Type I and Type III) are of special interest as only their derivatives are known to occur naturally. They are usually represented by the following structural formulas:[78]

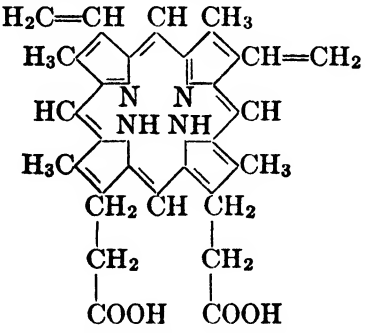


Etioporphyrin Type I



Etioporphyrin Type III

The porphyrin most closely related to naturally occurring heme is Type III Protoporphyrin. It may be derived from hemoglobin. It has also been combined with iron and protein to form hemoglobin and myoglobin.



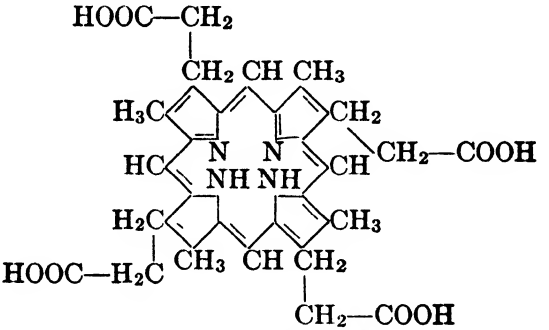
Protoporphyrin Type III

Coproporphyrin, $C_{36}H_{36}O_8N_4$, is a constituent of urine and feces. Its presence in the blood serum has been reported, as has its isolation from certain yeasts.

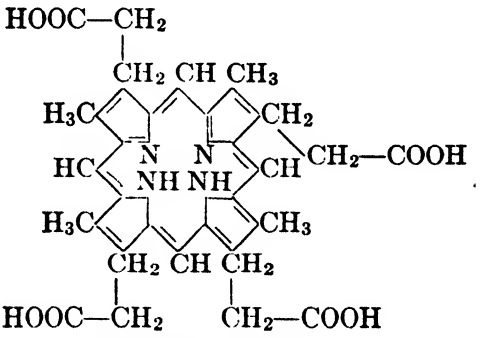
[77] The subject has been summarized by H. Fischer and H. Orth, in their book, "Die Chemie der Pyrrole. Volume II. Pyrrolfarbstoffe."

[78] Although the constitution of the porphyrins has been formulated by Hans Fischer, there is very little consistency in the way in which the structures of these compounds are represented by different writers. Compare H. T. Schreus, *Klin. Wochenschrift*, **13**, 121 (124); C. S. Leonard in Harrow and Sherwin's "Textbook of Biochemistry," Saunders, 1935, pp. 490–511; W. J. Turner, *J. Biol. Chem.*, **117**, 519 (1937).

From the urine of a normal individual coproporphyrin I has been isolated by Watson,[79] who believes that the type III compound is also present, but in much smaller amounts. According to Dobriner,[80] coproporphyrin I is the compound present in the feces of normal persons. The output, both in urine and feces, is markedly increased in certain pathological conditions. It is of interest that while the excretion of coproporphyrin I is affected in congenital porphyrinuria, hemolytic jaundice, pernicious anemia, cirrhosis due to cincophen poisoning, etc., in other disease processes (lead poisoning, atrophic and pigment cirrhosis), large amounts of coproporphyrin III are formed and excreted (Watson,[79] Dobriner [80]).



Coproporphyrin Type I



Coproporphyrin Type III

Dobriner and Rhoads [80] have submitted evidence that coproporphyrin I is excreted at a rate which is proportional to the rate of formation of protoporphyrin Type III compounds. The exact steps in the natural synthesis of coproporphyrin and protoporphyrin are unknown, but it is believed that they are formed from relatively simple " building stones," possibly from units composed of two pyrrole nuclei, united through a methene group.

[79] C. J. Watson, *J. Clin. Investigation*, **14**, 106, 110, 116 (1935); **15**, 327 (1936).

[80] K. Dobriner, *J. Biol. Chem.*, **113**, 1 (1936); *Proc. Am. Soc. Biol. Chem., ibid.*, **114**, xxvi (1936); *ibid.*, **120**, 115 (1937); Dobriner and C. P. Rhoads, *J. Clin. Investigation*, **17**, 95 (1938).

Uroporphyrin, $C_{40}H_{38}O_{16}N_4$ (Type I isomer), is excreted in the urine in congenital porphyrinuria. Rabbits poisoned with sulfonal may eliminate large amounts of uroporphyrin I. The copper salt of this compound is the pigment turacin present in the feathers of the turaco, a South African bird. The bones of the fox-squirrel, which are pink to dark red in color, contain uroporphyrin I. Large amounts of this pigment are likewise present in the urine and feces of this species of squirrel.[81] These are but a few of the many examples which may be cited to illustrate the widespread distribution and chemical relationship of the porphyrin derivatives in nature.

Heme and Its Derivatives. Corresponding to the fifteen protoporphyrins, an equal number of hemins is theoretically possible. Two have been synthesized, one being the natural derivative of hemoglobin. Similarly from the many other porphyrins, natural and synthetic, it is theoretically possible to prepare a series of hemes (and hemins) through their union with iron. This has been done in many cases; from mesoporphyrin and aetioporphyrin, mesohemin and aetiohemin have been prepared. Of the enormous number of possible hemes, we are, however, primarily concerned with only one, the heme of hemoglobin.

Heme combines with a large variety of organic nitrogenous substances to form a series of compounds that are now described as *hemochromogens*. These substances exhibit the same absorption spectrum pattern, but for individual members of the group, the absorption bands occupy somewhat different positions. The most familiar hemochromogen is derived from hemoglobin by the action of alkali and a reducing agent.[82] Hoppe-Seyler (1870) [83] thought that the product of this reaction was a substance represented by the formula $C_{34}H_{35}N_4O_4Fe$, and that it was this which gave the characteristic absorption spectrum. Accordingly the name "hemochromogen" was at first associated with this compound only. This conception was, however, erroneous.

Largely to the work of Anson and Mirsky [84] we owe a more correct understanding of the relationship between hemoglobin and hemochromogen. Several years before our knowledge of the heme compounds, as summarized in the preceding pages, had advanced to the present stage, they showed that when hemin is reduced in an alkaline solution, the resulting compound does not give the spectrum of hemochromogen. However, when globin is added the spectrum of hemochromogen appears. The work of these investigators, the details of which cannot be considered here, established that the substance which gave what until then had been called the hemochromogen spectrum was

[81] W. J. Turner, *J. Biol. Chem.*, **118**, 519 (1937).

[82] This reaction and the resulting absorption spectrum were first studied by the physicist, G. Stokes; *Proc. Roy. Soc.* (*London*), **13**, 355 (1863–4).

[83] *Ber.*, **3**, 229 (1870).

[84] *J. Physiol.*, **60**, 50, 161, 221 (1925); Anson, Barcroft, Mirsky, and Onuma, *Proc. Roy. Soc.* (*London*), *B*, **97**, 61 (1925); *J. Gen. Physiol.*, **12**, 273 (1928–29); *ibid.*, **12**, 581 (1929).

a conjugated protein, consisting of globin and the base $C_{34}H_{32}N_4O_4Fe$.

In hemoglobin, heme is attached to *native*, i.e., undenatured, globin; in globin-hemochromogen the combination is with *denatured* globin. There is no clear evidence that the two differ in other respects, as for example in their molecular weights, or in the manner in which the globin and heme are combined.

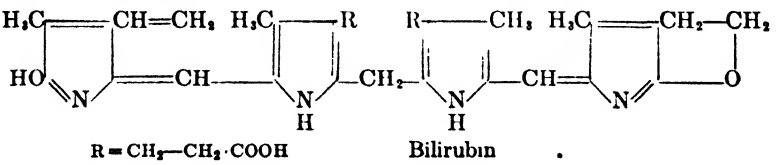
The synthetic hemochromogens include combinations of heme with albumin, pyridine, nicotine, piperidine, hydrazine, cyanide, ammonia, glycine, and other organic nitrogenous compounds. The relation of heme to these hemochromogens may be represented as follows:

$$\text{Heme} + \text{nitrogenous substance} \rightleftarrows \text{hemochromogen}$$

The various synthetic hemochromogens do not give identical absorption spectra, but they are sufficiently close to explain why the older workers, using less accurate spectroscopes, did not recognize the true nature of hemochromogen. In liberating the base from hemin, ammonia was often used. On subsequent reduction, instead of obtaining the reduced base, as was thought, a complex of this substance with ammonia, or as it would now be called, ammonia-hemochromogen, was formed. This substance gives an absorption spectrum which sufficiently resembles that given by the hemochromogen obtained directly from hemoglobin to have been mistaken for it.

Undenatured globin combines with heme to form crystallizable hemoglobin.[85]

Bilirubin. In another connection it was pointed out that bilirubin, $C_{33}H_{36}O_6N_4$, is formed in the destruction of hemoglobin in the cells of the reticulo-endothelial system, especially the liver. Bilirubin gives no absorption spectrum, from which it has been inferred that the porphyrin ring system is absent. It is now generally held that bilirubin is derived from hemoglobin by the opening of the ring of a type III porphyrin. The following formula has been proposed by Fischer and Haberland.[85a]



R = CH_2—CH_2·COOH Bilirubin

Urobilin, $C_{33}H_{42}O_6N_4$, has been isolated in crystalline form by Watson [86] and has been found to be identical with crystalline *stercobilin*.

[85] For further details the student is referred to a review by Anson and Mirsky, "Hemoglobin, the Heme Pigments and Cellular Respiration," *Physiol. Rev.*, **10**, 506 (1930); see also Anson and Mirsky, *J. Gen. Physiol.*, **14**, 605 (1931); R. Hill and H. Holden, *Biochem. J.*, **20**, 1326 (1926); *ibid.*, **21**, 625 (1927).

[85a] *Z. physiol. Chem.*, **232**, 236 (1935).

[86] C. J. Watson, *Z. physiol. Chem.*, **204**, 57 (1932); **208**, 101 (1932); **221**, 145 (1933); **233**, 39 (1935); *J. Biol. Chem.*, **105**, 469 (1934); **114**, 47 (1936). See also the review by Fischer and Orth, *Ann. Rev. Biochem.*, **3**, 410 (1934); Fischer and Halbach, *Z. physiol. Chem.*, **238**, 59 (1936).

It is an oxidation product of *urobilinogen*, $C_{33}H_{44}O_6N_4$, formed from bilirubin by reduction.

Helicorubin, Actiniohematin, Chlorocruorin, and Hemocyanin. *Helicorubin* is a respiratory pigment found in the liver and gut of the snail (*Helix pomatia*) and other pulmonate molluscs, as well as in the liver of the crayfish. It is a hemochromogen composed of globin and heme. Artificial hemochromogens prepared from this pigment are identical with those derived from hemoglobin. Helicorubin combines with oxygen, forming a compound capable of dissociation, thus resembling hemoglobin. However, it differs from hemoglobin in that its affinity for oxygen is greatest in a slightly acid medium.

Actiniohematin is a respiratory pigment, resembling helicorubin, which occurs in certain actinia. *Chlorocruorin* occurs in marine worms of the polychaete family. In concentrated solution it is reddish, whereas in dilute solution it has a green color. It may be oxidized and reduced like hemoglobin, which it resembles in other ways. Chlorocruorin yields derivatives corresponding to methemoglobin, hemochromogen, hematin, and hematoporphyrin. The artificial hemochromogens prepared from chlorocruorin, however, yield an absorption spectrum differing from the hemochromogen derived from hemoglobin, and it is therefore concluded that the porphyrin of chlorocruorin is different from protoporphyrin.

Hemocyanin is a copper-containing respiratory pigment found in certain crustaceans and molluscs. In the oxidized state it is blue; in the reduced state it is colorless. The hemocyanins derived from *Limulus polyphemus* (king-crab), and *Helix pomatia* (snail) are among the more familiar examples. Formerly hemocyanin was considered to be an analogue of hemoglobin, but this is unlikely inasmuch as there is no evidence for the existence of a copper-porphyrin nucleus in the molecule, as was supposed. The hemocyanin of *Octopus* has *p*H 4.8 as its isoelectric point; the hemocyanin of *Limulus* has its isoelectric point between 6.2 and 6.4. The hemocyanin of *Helix pomatia* may be readily crystallized; yet determinations of its molecular weight have yielded values ranging from 502,000 to 6,630,000, indicating that the compound may exist in varying degrees of aggregation. This seems to be true also of other hemocyanins; for example, the hemocyanin of *Busycon*, or winkle, a large marine snail, was found to have the following molecular weights (Svedberg [87]): 379,000, 6,814,000, and 9,980,000, the last representing the main or aggregation compound.

The Biological Significance of Heme Compounds; Cytochrome. If the occurrence of heme were limited only to hemoglobin, it would still be one of the most widely distributed and most important substances in nature. However, hemoglobin is not the only heme compound; there are others which are much more widely distributed. There exists an intracellular pigment in aerobic bacteria, yeast, higher plants, and ani-

[87] *Chem. Rev.*, **20**, 81 (1937).

mals. It was first observed in muscle and other tissues by MacMunn [88] in 1886 and named histohematin, but it is to the more recent work of Keilin [89] that we owe most of our knowledge of the subject. This pigment, renamed cytochrome, is capable of existing in an oxidized and in a reduced form. In the latter condition, it exhibits an absorption spectrum of four bands. Whatever the source of the cytochrome, these bands occupy approximately the same positions, namely, $a = 6046$; $b = 5665$; $c = 5502$; $d = 5210$, expressed in Angström units.

The work of Keilin has shown that cytochrome is not one substance, but a mixture of three independent hemochromogen-like compounds, a' b', c', capable of being oxidized and reduced independently from one another. In addition to these hemochromogens, or even in their absence, all cells of aerobic organisms contain a free unbound heme, which is apparently identical with the heme of hemoglobin.

The pigments designated collectively as cytochrome are found in highest concentration in cells capable of active metabolism. Heart muscle of mammals and birds, the pectoral muscle of flying birds, the thoracic muscles of flying insects, baker's yeast, and certain bacteria are among the active tissues that are especially rich in cytochrome. This striking relation of the concentration of pigment to tissue activity, as well as other experimental data, has led to the conclusion that cytochrome plays an important rôle in physiological oxidations, a view strengthened by recent reports that certain enzymes associated with cellular respiration (catalase, peroxidase, Warburg's so-called respiratory ferment) are derivatives of heme. In the succeeding chapter this phase of the subject will receive further attention.[90]

THE CHEMISTRY OF RESPIRATION

Mechanism for the Transportation of Oxygen. Hemoglobin is not a catalyst of oxidations, but a passive carrier of oxygen. The transportation of oxygen by the blood from the lungs to the tissues depends on a reversible chemical reaction between hemoglobin and oxygen, as represented by the equation:

$$Hb + O_2 \rightleftharpoons HbO_2$$

where Hb stands for hemoglobin.[91] Reduced hemoglobin is readily

[88] *Phil. Trans. Roy. Soc.*, **177**, 267 (1886).

[89] *Proc. Roy. Soc.* (*London*), *B*, **98**, 312 (1925); **100**, 206 (1928–29); *Nature*, **119**, 670 (1927).

[90] For comparatively recent reviews of the literature the reader is referred to A. E. Mirsky and M. L. Anson, "Animal Pigments," *Ann. Rev. Biochem.*, **3**, 425 (1934), and J. Roche, *ibid.*, **5**, 463 (1936).

[91] Adair (*J. Biol. Chem.*, **63**, 529 [1925]) has attempted to explain the equilibrium between oxygen and hemoglobin on the assumption that the latter combines with oxygen in steps to form Hb_4O_2, Hb_4O_4, Hb_4O_6, and Hb_4O_8. The equilibrium relations between oxygen and hemoglobin have also been studied recently by Ferry and Green (*J. Biol. Chem.*, **81**, 175 [1929]) and by Conant and McGrew (*ibid.*, **85**,

oxidized to oxyhemoglobin when exposed to oxygen of such concentration as exists in the lungs; oxyhemoglobin is, in turn, dissociated at low oxygen tensions, such as obtain in the tissues. These reactions occur with extreme rapidity, requiring but a fraction of a second, as has been shown by Hartridge and Roughton.[92] It should also be mentioned at the outset that not all the hemoglobin is oxidized in the lungs, nor is all the oxygen given up in the tissues.

In a mixture of gases, each gas exerts its own partial pressure. The oxygen content of the air at sea level is about 21 per cent. From this it follows that the partial pressure of the oxygen in the air is about 160 mm. of mercury, when the atmospheric pressure is 760 mm. In the alveoli of the lungs the oxygen content is only about 14 per cent; this is equivalent to approximately 106 mm. of mercury. Ordinarily, this is the maximum oxygen tension to which the hemoglobin of the blood is exposed in the course of its circulation.

One liter of plasma saturated with alveolar air takes up about 3 cc. of oxygen. The oxygen capacity of the blood is about 1 liter, this amount of oxygen being ordinarily sufficient for tissue needs. If we were dependent, therefore, upon the solubility of oxygen in the blood alone, our circulatory system would have to contain about 300 liters of fluid or about four times our body weight. Owing to the presence of hemoglobin the enormous quantity of oxygen which we need is handled by about 6 liters of blood. As stated by Barcroft, "the warm-blooded creation owes its existence, or at all events its activity, to hemoglobin."[93]

In passing through the capillaries of the lungs, the blood is exposed to an oxygen tension of about 100–110 mm. of mercury. As it leaves

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421 [1929–30]). The latter workers found that if solutions of oxyhemoglobin are deoxygenated, the Hb_4O_8 persists and does not disappear as would happen if it were converted into the intermediate products (Hb_4O_6, Hb_4O_4, Hb_4O_2), which have much higher solubilities than the fully oxidized hemoglobin. Conant and McGrew have suggested that, if intermediate oxidation products of hemoglobin are formed, they are present in very small quantities.

[92] *Proc. Roy. Soc.* (*London*), *A*, **104**, 395 (1923).

[93] In man, and perhaps in other animals, the quantity of hemoglobin appears to be regulated by the demand for oxygen and its supply. Thus, at high altitudes, where the amount of oxygen is reduced, the quantity of hemoglobin in the blood is increased. A striking illustration of this is to be found in the observations of Barcroft and his associates, who studied the blood of the natives in the Cerro de Pasco region of the Peruvian Andes, 14,000–15,000 feet above sea level. Of twelve cases studied, one had 150 per cent of the normal amount of hemoglobin; three had from 140–149 per cent; four gave hemoglobin values from 130 to 139 per cent; and the remaining four had from 120 to 129 per cent of the normal amount observed in man at sea level. *Phil. Trans. Royal Soc.*, *B*, **211**, 351 (1922–23). A fascinating account of the expedition to Cerro de Pasco is given in J. Barcroft's "The Respiratory Function of the Blood," Part I, Lessons from High Altitudes, 2d Edition, Cambridge, 1925.

A. Grollman, *Am. J. Physiol.*, **93**, 19 (1930), in a series of determinations found a steady increase in hemoglobin to 140 per cent during the first two weeks of a prolonged stay on Pike's Peak (altitude, 14,109 feet).

the lungs, the blood (i.e., arterial blood) has an oxygen content of about 19 volumes per cent; the tension is about 80 mm. of mercury; the percentage saturation of the hemoglobin is 93–98 per cent.[94] In the tissues the oxygen tension is much lower, perhaps of the magnitude of 0–10 mm. of mercury.[95] Consequently when the blood reaches the tissues, a part of the oxyhemoglobin is dissociated, and, as the oxygen is liberated, there is a progressive fall in the oxygen tension of the blood. The blood returning from the tissues to the lungs, i.e., venous blood, has an oxygen content about 15 volumes per cent and a tension of about 40 mm. of mercury; the percentage saturation of the hemoglobin is usually 60–70 per cent. The liberated oxygen passes from the blood to the lymph and from the lymph to the tissues, and there takes part in the processes of oxidation, which we shall consider in succeeding chapters.

The transfer of oxygen in the lungs may be accounted for solely on the basis of diffusion. Secretion of oxygen by the alveolar epithelium does not occur, even at high altitudes, as shown by the earlier work of Krogh and of Barcroft and more recently confirmed by the observation of Dill, Christensen, and Edwards[96] in their high-altitude expedition to the Chilean Andes.

Factors Influencing the Combination of Hemoglobin and Oxygen. *Effect of Temperature.* As is true of many other chemical combinations, the union of oxygen and hemoglobin is less the higher the temperature. Thus, when blood is allowed to come to equilibrium with oxygen at a tension of 100 mm. of mercury, 93 per cent will become saturated at 38° C., and 98 per cent at 25° C. Under a pressure of 10 mm., at a temperature of 38° C., 56 per cent of the hemoglobin will still be in combination with oxygen, whereas at 25° C. the amount in combination will be 88 per cent. This means that, with a drop in oxygen tension commensurate with the difference between the pressure in the lungs and in the tissues, the amount of oxygen that becomes available for purposes of metabolism is greater at the higher than at the lower temperature. From this point of view, the advantage of being a warm-blooded animal is fairly obvious. These relationships are more completely illustrated in the accompanying diagram (Fig. 26) in which are presented a number of oxyhemoglobin-dissociation curves obtained at different temperatures.

Effect of Altitude. As regards its affinity for oxygen, hemoglobin produced during acclimatization to high altitudes does not appear to differ from that produced at sea level (Hall[97]).

Effect of Electrolytes. At low oxygen tensions, oxyhemoglobin is

[94] These are approximately the values obtained in the resting subject. Compare L. J. Henderson, "Blood, A Study in General Physiology," pp. 195, 201. For the changes occurring during work, see Chapter IX.

[95] Much higher values have been cited, namely 50–20 mm (or less) for extracellular tissue fluid and 40–20 mm. for the oxygen tension within the cell. J. A. Campbell, "Gas Tension in the Tissues," *Physiol. Rev.*, **11**, 1 (1931).

[96] *Am. J. Physiol.*, **115**, 530 (1936).

[97] *J. Biol. Chem.*, **115**, 491 (1936).

more readily dissociated in the presence of salts than in pure solution. If the temperature is maintained constant at 38° C., the saturation of hemoglobin in the presence of electrolytes may be reduced to less than one-half of what it is in pure solution, at an oxygen tension of 10 mm. of mercury. That this effect is not obtained at higher pressures is shown by the curves in Fig. 27, where an increase in the combining capacity of the hemoglobin is actually indicated at 100 mm.

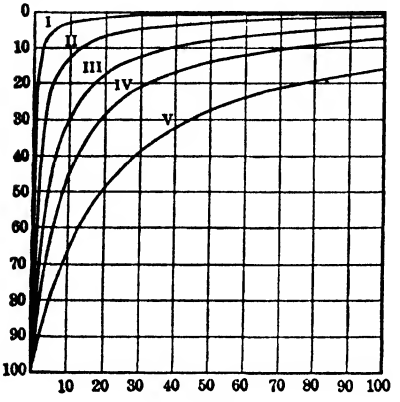


FIG. 26

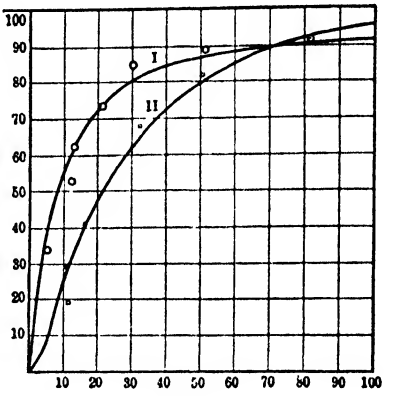


FIG. 27

FIG. 26.—Dissociation curves of oxyhemoglobin at different temperatures. Curves I, II, III, IV, and V correspond to 16°, 25°, 32°, 38°, and 49° C., respectively. Ordinates = percentage of reduced hemoglobin; abscissas = tension of oxygen in millimeters of mercury. (After Barcroft and Hill, *J. Physiol.*, **39**, 422 [1909–10].)

FIG. 27.—Effect of electrolytes on the dissociation curve of oxyhemoglobin. Ordinates = percentage saturation of hemoglobin with oxygen; abscissas = tension of oxygen in millimeters of mercury. ⊙ Points determined from dialyzed solution. ° Points determined from undialyzed solution. Curve I (electrolytes absent) = rectangular hyperbola; $xy = 800$. Curve II (electrolytes present in low concentration) = Bohr's dissociation curve for hemoglobin (see *Zentr. Physiol.*, **17**, 682, 688 [1903–04]). (After Barcroft and Roberts, *J. Physiol.*, **39**, 146 [1909–10].)

Effect of Carbon Dioxide. A third factor influencing the efficiency of hemoglobin as a carrier of oxygen is carbon dioxide. In view of the acidity of carbonic acid, the effect of carbon dioxide may be referred to the hydrogen-ion concentration changes. This relationship has been studied by Barcroft and Poulton [98] and others. Bock, Field, and Adair [99] obtained oxygen-dissociation curves for normal blood at carbon-dioxide tensions of 3, 20, 40, and 80 mm. of mercury. From their results, some of which are reproduced below (Fig. 28), it may be seen that carbon dioxide, while not hindering, to any appreciable extent, the formation of oxyhemoglobin in the lungs, greatly facilitates its dissociation in the tissues. In the removal of carbon dioxide from the

[98] *J. Physiol.*, **46**, iv (1913).
[99] *J. Biol. Chem.*, **59**, 353 (1924).

tissues, which is intimately associated with its rôle in the transportation of oxygen, hemoglobin plays an important part. This function will be discussed presently.

The isoelectric point of oxyhemoglobin as given by Adair is 6.6, and of reduced hemoglobin, 6.81. On the acid side of the isoelectric point of hemoglobin its affinity for oxygen is less than on the alkaline side. The oxyhemoglobin dissociation curves obtained at various hydrogen-ion concentrations have been described by Adair.[100]

Mechanism for the Transportation of Carbon Dioxide. The carbon dioxide content of atmospheric air is about 0.02 to 0.03 per cent, corresponding to a tension which is negligible (0.15 to 0.23 mm. of mercury). At rest the alveolar air contains about 5.5 per cent carbon dioxide, equivalent to a tension of 40–42 mm. This is essentially the same in arterial blood, which, however, has a total carbon dioxide content of 45 to 50 volumes per cent. Venous blood contains 50 to 55 volumes per cent of carbon dioxide and the pressure is about 45–47 mm. of mercury.[101] Presumably the carbon dioxide tension is somewhat above this value in the intracellular and extracellular fluid of the tissues. In short, if diffusion plays an important part in gaseous exchange, as it evidently does, the direction for carbon dioxide would be from the tissues to the blood and in turn to the lungs and the outside air.

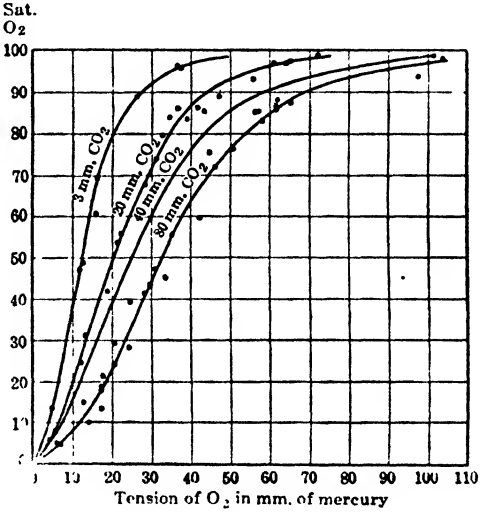


FIG. 28.—Effect of carbon dioxide on the dissociation of oxyhemoglobin. (After Bock, Field and Adair.)

Does Hemoglobin Combine with Carbon Dioxide? The relatively large amounts of carbon dioxide carried in the blood at the correspond-

[100] *Ibid.*, **63**, 529 (1925).

[101] Data are given by Henderson (p. 259) comparing the pCO_2 (CO_2 tension in millimeters of mercury) of the blood at work and at rest (subject A. V.).

	Arterial	Venous
Work	38	54 8
Rest	40	45 1

ing low tensions indicate that the greater proportion is not in solution, but in some form of combination. Considering also that the transportation of oxygen depends almost exclusively on its reversible chemical union with hemoglobin, it is logical to inquire whether a similar combination does not occur with carbon dioxide. Indeed such a reaction was assumed by Bohr [102] in 1905.

Until recently all theories of carbon dioxide transport which, like Bohr's, assumed combination with hemoglobin, received scant notice, while the predominant opinion of the transport of carbon dioxide almost exclusively as bicarbonate and carbonic acid in a ratio approximating 20 : 1 seemed to be securely established. Indeed, a skeptical attitude toward the latter view did not develop until after it was shown by Henriques [103] that the rate of liberation of carbon dioxide in the lungs was much too rapid to be explained simply on the basis of a decomposition of bicarbonate. This reaction involves the intermediate formation of carbonic acid, the dehydration of which is a comparatively slow process even in serum. It was precisely this feature of the reaction which stimulated further inquiry and led to the observation that the presence of erythrocytes greatly accelerates the release of anhydrous carbon dioxide. At the time, there seemed to be two possible explanations of this phenomenon: (1) that the red cells contained a catalyst for the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$; and (2) that it was due to a rapidly reversible reaction between hemoglobin and CO_2 of a direct type, analogous to the oxygen-hemoglobin reaction.

Both these explanations have since been subjected to critical investigation. Some of the earlier results seemed to indicate that hemoglobin itself possessed considerable activity in accelerating the rate of carbonic acid dehydration, but further study disclosed that the hemoglobins of certain organisms did not exert this effect. Eventually the active principle was found to be in the nature of an enzyme, and its isolation from red blood cells was accomplished independently by two groups of workers, Meldrum and Roughton [104] in England, and Stadie and O'Brien [105] in the United States. Because of its behavior the enzyme has been named *carbonic anhydrase*. According to Roughton,[106] there is sufficient carbonic anhydrase in red cells to accelerate the reaction about 1500 times at 38° C. As this is approximately ten times the necessary acceleration, it may be guessed that the enzyme is present well in excess of the minimal requirements.

The action of the enzyme is completely inhibited in the presence of cyanide. Hemoglobin solutions saturated with carbon dioxide, to

[102] C. Bohr, in Nagel's "Handbuch der Physiologie," **2**, 106 (1905); Bohr's theory is also discussed in J. S. Haldane's "Respiration," p 87, Yale Univ. Press, New Haven, 1922.

[103] *Biochem. Z.*, **200**, 1 (1928); *Ergeb. Physiol.*, **28**, 624 (1929).

[104] *Proc. Roy. Soc. (London)*, **111**, 296 (1932); *J. Physiol.*, **75**, Proc., 3p.(1932).

[105] *J. Biol. Chem.*, **100** (*Proc.*), lxxxviii (1933); **103**, 521 (1933).

[106] *Physiol. Rev.*, **15**, 241 (1935).

which cyanide has been added, are nevertheless capable of releasing and combining with CO_2. This observation lends support to Henriques' view of a reaction between hemoglobin and carbon dioxide. The nature of the combination has been determined by Roughton and fellow-workers and by Stadie and O'Brien. It seems that the carbon dioxide is bound to the hemoglobin through an amino group, forming a carbamino derivative:

$$Hb \cdot NH_2 + CO_2 \rightleftharpoons Hb \cdot NH \cdot COOH \rightleftharpoons Hb \cdot NH \cdot COO^- + H^+$$

State of Carbon Dioxide in the Blood. The carbamino-bound CO_2 constitutes less than 10 per cent of the total CO_2 of the blood, but its physiological importance in the respiratory cycle is proportionately greater, as indicated by the fact that approximately 20 per cent of the difference in CO_2 between arterial and venous blood is due to the difference in carbamino-bound CO_2. In the red cells about 40 per cent of the change in CO_2 content during the respiratory cycle has been estimated as being due to changes in carbamino-bound CO_2.

According to Roughton,[106] of the total CO_2 liberated from the blood in passing through the lungs (subject at rest) about

70 per cent comes from bicarbonate ions,
10 per cent comes from preformed dissolved CO_2, and
20 per cent comes from the carbamino compound of CO_2 with hemoglobin.

Owing to the presence of carbonic anhydrase the bicarbonate which is present in the red cells reaching the lung is quickly dissociated and the liberated CO_2 migrates outward through the cell membrane into the plasma and thence into the alveoli of the lung. In contrast, of the bicarbonate in the plasma the amount of CO_2 liberated *directly* is insignificant, and yet approximately 50 per cent of the total CO_2 evolved in the lungs comes from this source. This is brought about by the migration of bicarbonate ions from the plasma into the corpuscles, a process involving an exchange with chloride ions which pass outward. Within the red cell the bicarbonate rapidly dissociates, through the action of carbonic anhydrase and the liberated CO_2 is returned to the plasma and released in the lung. These changes may be represented graphically, as in Fig. 29.

The difference between the total bound CO_2 and the CO_2 present in the form of free bicarbonate represents the CO_2 in other kinds of combination. This has been designated by Roughton as x-bound CO_2, of which carbamino-bound CO_2 is the principal component. Perhaps the only other compound is so-called y-bound CO_2, believed by Roughton to be hemoglobin bound directly with bicarbonate ions. There is no evidence that this substance is of any importance in the respiration of mammals.

Summarizing our present knowledge, it may be stated that the greater proportion of the blood CO_2 occurs as bicarbonate in combination with base (chiefly Na and K). Smaller amounts are present as preformed H_2CO_3 and dissolved CO_2. The remainder, constituting 10 per cent or less of the total, is present in combination with hemoglobin

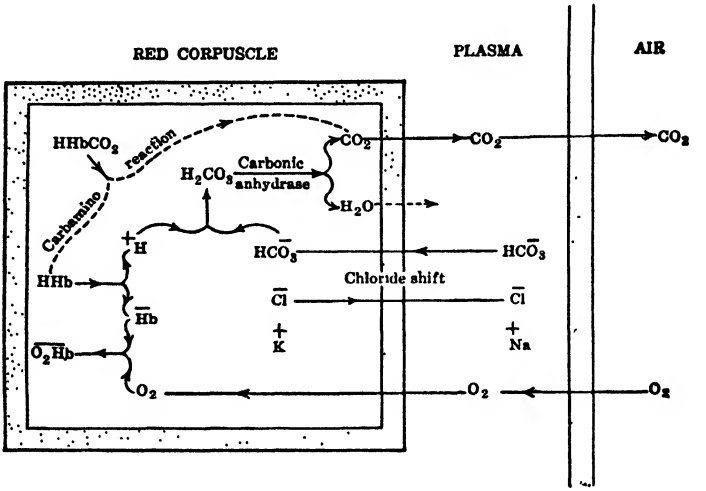


FIG. 29.—Schematic survey of main processes occurring with the blood, during CO_2 output. Reproduced through courtesy of F. J. W. Roughton.

as carbamate. An idea of the approximate partition of CO_2 in plasma and cells of arterial and venous blood may be gained from the data in Table XXXIII.

TABLE XXXIII

PARTITION OF CARBON DIOXIDE IN PLASMA AND CELLS AT 38° C. IN CUBIC CENTIMETERS PER 100 CC. OF BLOOD*

	Arterial			Venous		
	Plasma	Cells	Whole Blood	Plasma	Cells	Whole Blood
Total CO_2	35 7	12.5	48 2	38 0	14 0	52.1
Free CO_2	1.6	0 8	2 4	1 8	0 9	2.7
Bound CO_2	34.1	11 7	45 8	36 3	13.1	49.4
CO_2 as bicarbonate	33 1	9 8	42.9	35 2	10 5	45 7
CO_2 as carbamate	1 0	1 9	2 9	1.1	2.6	3.7

* Based on the data of W. C. Stadie and H. O'Brien, *J. Biol. Chem.*, **117**, 439 (1937).

Owing to the partition of carbon dioxide in various forms, extremes of acidity and alkalinity are prevented. As was pointed out by Van

Slyke [107] long ago, if all the CO_2 were present as H_2CO_3, the blood would be a thousand times more acid than it is. If all the CO_2 were present as bicarbonate, the blood would be hundreds of times too alkaline. There is normally a definite balance or ratio between the amount of CO_2 present as H_2CO_3 and the amount present as $BHCO_3$. Because there is a mechanism which makes this balance possible, the blood is able to carry large amounts of carbon dioxide from the tissues to the lungs, to be excreted, without the production of any very marked change in its hydrogen-ion concentration. We shall now examine the nature of this mechanism.

The Nature of Buffers. Buffer action may be defined as the resistance to change in hydrogen-ion concentration. Solutions may be prepared of mixtures of acids or bases with an excess of alkali salts in the case of the acids, and, in the case of the bases, an excess of their salts with strong acids. Such solutions, termed "buffer solutions," are distinguished by the fact that dilution, or the addition of moderate amounts of acid or base, has but little effect upon their hydrogen-ion concentration.

Buffer action may be illustrated by the following example. If, to a liter of pure water of *p*H 7.0, we should add 1 cc. of 0.01 *N* hydrochloric acid, the hydrogen-ion concentration of the resulting solution would become equivalent to a *p*H of about 5.0. If, however, instead of using pure water, we should add the same amount of acid to water containing potassium acid phosphate (KH_2PO_4) and potassium hydroxide in such proportion as to give the solution a *p*H value of 7.0, the resulting change in hydrogen-ion concentration would be hardly measurable.

The behavior of sodium acetate as a buffer is similar. When added to acetic acid, sodium acetate reduces the ionization of the acid and consequently the C_H of the solution. If, to a solution of acetic acid and sodium acetate, hydrochloric acid is added, little effect is produced on the hydrogen-ion concentration, as the hydrogen ions combine with acetate ions to form the weakly ionized acetic acid. On the other hand, the same amount of hydrochloric acid added to water or to a solution of sodium chloride would cause a marked increase in hydrogen ions. The sodium acetate-acetic acid solution would also be effective against hydroxyl ions. The addition of a base like sodium hydroxide would result in its reaction with the acetic acid to give sodium acetate.

Fernbach and Hubert,[108] who first studied the power of certain solutions to resist changes in reaction, compared their action to that of a tampon. Sørensen translated this by the German word "Puffer." In translating this into English, the word "buffer" has been adopted. Various analogies have been employed to explain buffer action. A buffer has been compared to a sponge having the capacity of "soaking up" hydrogen and hydroxyl ions. It has also been likened to a shock

[107] *Physiol. Rev.*, **1**, 141 (1921).
[108] *Compt. rend.*, **131**, 293 (1900).

absorber. Just as a shock absorber blocks the transmission of the full force of an impact, so a chemical buffer resists the change in H^+ ion concentration which tends to occur when acid or alkali is added.

The Buffers of the Blood. The buffers of the blood are salts of weak acids. These are the bicarbonates, phosphates, and alkali salts of the proteins, including both hemoglobin and oxyhemoglobin. In each case, however, part of the buffer is present as the free acid, the remainder as the salt of the weak acid with a strong base. We thus have a number of what we might term buffer pairs. These are:

$$\frac{H_2CO_3}{BHCO_3'} \quad \frac{BH_2PO_4}{B_2HPO_4'} \quad \frac{HHbO_2}{BHbO_2'} \quad \frac{HHb}{BHb'} \quad \frac{\text{H protein}}{\text{B protein}}$$

B is used here to indicate any monovalent base such as sodium or potassium, $HHbO_2$ the free oxyhemoglobin, $BHbO_2$ the alkali salt of oxyhemoglobin, HHb the free or acid hemoglobin, BHb the basic salt of hemoglobin, H protein the free protein, and B protein the alkali proteinate, respectively.

The maintenance of the acid-base balance of the blood is not dependent upon any one buffer pair, but rather upon the total effect of several such pairs. The advantage of such an arrangement in providing security against acidosis or alkalosis may be likened to the advantage of having five sentinels on duty instead of one. It is obviously unsafe to leave a single individual to safeguard a treasure or to hold and defend a mountain pass against an enemy. No matter how well armed he may be, he is always in great danger of being overcome. Five good guards strategically placed would constitute a much more solid defense. If one or two of the guards were overcome there would still be some left to hold the enemy back for a time, perhaps even until help arrived. This analogy, crude as it may be, is quite apropos if we suppose the enemy to be H^+ or OH^- ions, and the mountain pass the outer limits of the normal *p*H range of the blood. Of course, all five guards in our analogy might be overcome. Translated into terms of acid-base balance, this is what happens when severe acidosis or alkalosis develops.

General Laws. There are two general laws which may be applied to buffer solutions.[109]

1. The hydrogen-ion concentration of a buffer solution is proportional to the ratio $\frac{\text{free buffer acid}}{\text{buffer salt}} = \frac{HA}{BA'}$, where A represents the acid radical and B a monovalent base.

2. A given buffer mixture is most efficient in maintaining constancy of *p*H when the ratio $\frac{HA}{BA}$ is equal to 1, and when H^+ approximates K, the dissociation constant of the free acid forming one of the buffer pairs.

[109] L. J. Henderson, *Ergeb. Physiol.*, 8, 254 (1909); see also Van Slyke.[107]

Relationship between pH and the Ratio $\frac{HA}{BA}$. The proof of the first law will be considered at this point. The dissociation of an acid HA may be represented by the equation $HA = H^+ + A^-$. From the law of mass action, it follows that, when equilibrium is reached,

$$K \times HA = H^+ \times A^- \tag{1}$$

where K is the dissociation constant of the acid. Therefore,

$$H^+ = K\frac{HA}{A^-} \tag{2}$$

However, in buffer mixtures of the type with which we are concerned, there is to be considered not only the weak acid, but the salt of the acid as well. No matter which of the buffer pairs we may select, the dissociation of the free acid is negligible as compared with the dissociation of the salt BA (salt of a weak acid and a strong base). It is to be noted here that the salts are present in the blood in low concentration, which means that they are, relatively speaking, highly dissociated (60–90 per cent). The degree of dissociation, which may be represented by λ, will not vary appreciably for any given base over the range of its concentration in the blood. The concentration of the anions A^- is therefore equal to λBA. Substituting in equation (2),

$$H^+ = K\frac{HA}{\lambda BA} \tag{3}$$

As λ remains practically constant, the above equation may be simplified by substituting K_1 for K/λ. Thus,

$$H^+ = K_1\frac{HA}{BA} \tag{4}$$

Expressed in terms of pH, equation (4) becomes

$$p\mathrm{H} = -\log K_1 - \log\frac{HA}{BA} \tag{5}$$

The symbol pK_1 may be used to signify the logarithm of the reciprocal of K_1. Hence equation (5) may be written

$$p\mathrm{H} = pK_1 + \log\frac{BA}{HA}. \tag{6}$$

For any given buffer pair, pK_1 remains practically constant. It is 6.1 for $BHCO_3 : H_2CO_3$ (Hasselbach corrected by Van Slyke). For the phosphates it is 6.8. By substituting these values for pK_1 in equation (6), it becomes possible to calculate the ratio of a buffer mixture for any given pH, or, if the ratio is known, the pH may be calculated.

Efficiency of Buffers. According to the second generalization, the maximum efficiency of a buffer is obtained when $\frac{BA}{HA} = 1$, and $[H]^+ = K_1$. The bicarbonate : carbonic acid ratio is 1 at a *p*H of 6.1, while at a *p*H of 7.4 it is equal to $\frac{20}{1}$. Offhand it may be supposed that the bicarbonate, as a buffer, does not act with its maximum efficiency in the blood, where the *p*H is about 7.35. However, owing to the fact that the solubility of carbonic acid in water is limited to 2.75 volumes per cent, the generalization applying to the efficiency of buffers does not hold in this particular case. And indeed the maximum efficiency of this buffer mechanism is at the *p*H of the blood (7.35). At the same *p*H the ratio $Na_2HPO_4 : NaH_2PO_4$ is 3.55, whereas at 6.8 the ratio is 1. This means that the closer the reaction of the blood approaches the danger zone, the more efficient is the buffer action of the phosphates.

Two factors contribute to the buffer mechanism of the blood. The first is the buffer action of the bicarbonate and proteins of the plasma, and of the bicarbonate, phosphates, and proteins of the cells. The second factor depends upon the property of hemoglobin to change from a weak acid to a relatively strong one when it changes from the reduced to the oxidized form. The dissociation constant of reduced hemoglobin (of the horse) is approximately 1/29 of that of oxyhemoglobin.

Neutralization of Acid and the Transportation of Carbon Dioxide. All buffers participate in the neutralization of acid. Let us assume that a large amount of lactic acid is being formed which requires neutralization. The sodium bicarbonate will react with it as follows:

$$NaHCO_3 + \text{lactic acid} = Na\cdot\text{lactate} + H_2CO_3$$

The basic phosphates would react similarly:

$$B_2HPO_4 + \text{lactic acid} = B\cdot\text{lactate} + BH_2PO_4$$

The plasma proteins would neutralize a portion according to the equation:

$$B\cdot\text{protein} + \text{lactic acid} = B\cdot\text{lactate} + H\cdot\text{protein}$$

The remaining buffers would act in the same way.

The maintenance of the reaction of the blood at a constant level and the transportation of carbon dioxide are both due to the giving up, by the buffer salts, of part of their reserves of alkali for the purpose of neutralizing any acid, including carbonic acid. There is therefore an intimate connection between the buffer action of certain substances and their rôle as carriers of carbon dioxide. According to the definition given by Van Slyke, a carbon-dioxide carrier is a constituent of the blood that increases the amount of carbon dioxide which may be taken up by arterial blood with a change in reaction equal only to the normal *p*H difference between arterial and venous blood.

All buffers act in essentially the same manner as carriers of carbon dioxide. For the purpose of illustration, we shall consider the behavior of the phosphates and calculate the changes that occur when the H_2CO_3 is increased sufficiently to lower the *p*H from 7.35 to 7.25. *Of course, it is to be clearly understood that changes of this magnitude do not actually occur in the blood.* This marked shift, which is several times the normal *p*H difference between arterial and venous blood, is selected mainly in order to make the calculations somewhat more striking and the illustration somewhat clearer.

In these calculations it will be assumed that the concentration of total phosphate ($Na_2HPO_4 + NaH_2PO_4$) is 0.05 *M*; and of $NaHCO_3$, 0.03 *M*.

Recalling the relation between the ratio of a buffer pair and *p*H, and substituting 6.80 for pK_1 in the equation representing this relation, we have

$$\log \frac{Na_2HPO_4}{NaH_2PO_4} = 7.35 - 6.80 = 0.55$$

or

$$\frac{Na_2HPO_4}{NaH_2PO_4} = 3.55$$

As the total concentration of PO_4 is 0.05 *M*, the concentration of NaH_2PO_4 is obviously 0.05 − Na_2HPO_4. Calculating, we find that at *p*H 7.35,

$$\frac{Na_2HPO_4}{NaH_2PO_4} = \frac{0.0390}{0.0110} = 3.55$$

The ratio changes with *p*H, being but 2.82 at *p*H 7.25. Calculating as before, we find that at *p*H 7.25,

$$\frac{Na_2HPO_4}{NaH_2PO_4} = \frac{0.0369}{0.0131} = 2.82$$

That the change in *p*H is accompanied by the release of a certain amount of base can be seen from the fact that, in the equation above, NaH_2PO_4 has one less sodium than Na_2HPO_4. The amount of alkali which is thus set free to combine with H_2CO_3 to form bicarbonate is calculated as follows:

$$\begin{array}{r} 0.0390\ M\ Na_2HPO_4 \\ -\ 0.0369\ M\ Na_2HPO_4 \\ \hline \text{Difference} = 0.0021\ M\ \text{Na set free to form } NaHCO_3. \end{array}$$

We began with an initial bicarbonate concentration of 0.03 *M*. At the higher *p*H,

$$\log \frac{NaHCO_3}{H_2CO_3} = 7.35 - 6.10 = 1.25$$

hence

$$\frac{NaHCO_3}{H_2CO_3} = 17.8$$

It therefore follows that

$$H_2CO_3 = \frac{0.03}{17.8} = 0.00169\ M$$

At *p*H 7.25 the ratio $NaHCO_3 : H_2CO_3$ is equal to 14.1. Had the $NaHCO_3$ concentration remained constant, the H_2CO_3 would have been

$$H_2CO_3 = \frac{0.03}{14.1} = 0.00212\ M$$

This is not the case, however, for it has just been shown that, with the change in *p*H from 7.35 to 7.25, the $NaHCO_3$ concentration increased to 0.0321 *M*. Therefore, at *p*H 7.25,

$$H_2CO_3 = \frac{0.0321}{14.1} = 0.00228\ M$$

The difference between 0.00212 and 0.00169 = 0.00043 *M* represents the amount of free H_2CO_3 that is added to 0.03 *N* $NaHCO_3$ solution with a change in *p*H from 7.35 to 7.25. The difference between 0.00228 and 0.00212 = 0.00016 *M* represents the amount of free H_2CO_3 added because of the buffer effect of the phosphates and the consequent increase of the $NaHCO_3$ concentration. Thus, the additional amount of carbon dioxide that can be carried on account of the presence of phosphates is

$$\begin{aligned} &0.00210\ M\ CO_2 \text{ as } NaHCO_3 \\ +\ &0.00016\ M\ CO_2 \text{ as } H_2CO_3 \\ +\ &\overline{0.00226\ M\ CO_2} = CO_2 \text{ capacity of the phosphates} \end{aligned}$$

between *p*H 7.35 and 7.25.

To avoid misconceptions it is again emphasized that the foregoing calculations are intended merely to illustrate the manner in which the buffers of the blood contribute their quotas of base, in order that the removal of carbon dioxide may proceed at a more or less constant rate. In reality, the phosphates play a very minor rôle as carriers of carbon dioxide, the amount of base liberated from this source in the tissues in a normal respiratory cycle representing less than 0.5 per cent of the total. Of somewhat greater importance is the buffer effect of the plasma proteins which release sufficient base for the removal of perhaps 5 to 10 per cent of the carbon dioxide.

Most important of all is hemoglobin, which in the transportation of carbon dioxide plays a triple rôle. One is the formation of the hemoglobin-carbamino compound, the significance of which in the respiratory cycle has been stated (p. 257). Hemoglobin further contributes to the carbon-dioxide capacity of the blood by virtue of its buffer action, that is through change in *p*H and direct competition of H_2CO_3, Hb, and HbO_2 for base. But of even more importance is the fact that about half (53 per cent according to Doisy, Briggs, Eaton and Chambers[110]) of the carbon dioxide is carried in combination with the base liberated as a result of the change in oxygenation of the hemoglobin in the tissues and lungs. This is the so-called *isohydric* change of oxyhemoglobin to hemoglobin:

$$BHbO_2 \rightleftarrows HHb$$

The explanation for this lies in the fact that oxyhemoglobin is a stronger acid than reduced hemoglobin, as evidenced by the following values for their dissociation constants:[111]

$$\begin{aligned} K \text{ for oxyhemoglobin} &= 2.4 \times 10^{-7} \\ K \text{ for reduced hemoglobin} &= 6.6 \times 10^{-9} \end{aligned}$$

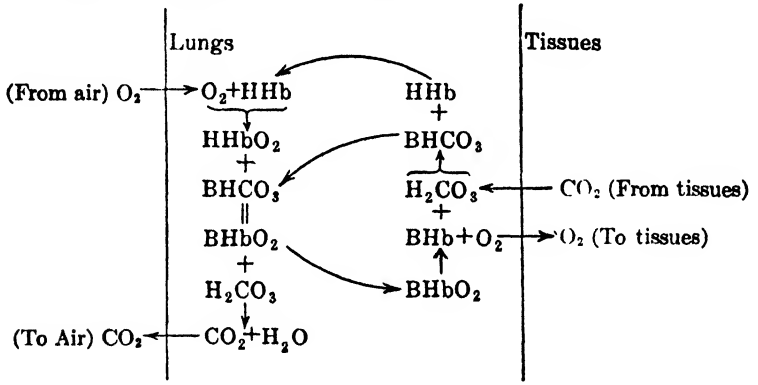
[110] *J. Biol. Chem.*, **54**, 305 (1922).

[111] These values are based on data given by Hastings, Sendroy, Murray, and Heidelberger, *J. Biol. Chem.*, **61**, 317 (1924). See also *ibid.*, **60**, 89 (1924).

Both the reduced and the oxidized forms exist partly as free acids and partly as the salts of strong bases. The tendency of oxyhemoglobin to combine with base is greater, however, than that of reduced hemoglobin, and hence, at a given *p*H, the proportion of salt to acid will be greater in the former case than in the latter.

$$\frac{BHbO_3}{HHbO_3} > \frac{BHb}{HHb}$$

This is very significant, for, in changing to the reduced form in the tissues, oxyhemoglobin liberates sufficient base to neutralize a considerable part of the carbon dioxide that is present. Subsequently, when the reduced hemoglobin reaches the lungs and is oxidized, it reacts with bicarbonate, with the consequent liberation of carbon dioxide. Exclusive of the hemoglobin-carbamino mechanism (p. 258), the cycle may be represented as follows:



That the most important buffer of the blood is isolated within the corpuscles is a matter worthy of note.

Buffer Effect of Plasma and Corpuscles; Chloride Shift. When plasma or serum is in contact with red cells its capacity for taking up carbon dioxide is much greater than in the absence of cells. This is because the cells pass on their buffer effect to the plasma in accordance with the mechanisms that have been discussed. Some features still remain to be considered, however.

Zuntz [112] found that the titratable alkalinity of serum could be increased by subjecting the blood to high tensions of carbon dioxide. He concluded that the carbonic acid passed into the corpuscles, where it split off alkali from the cell proteins, and that the alkali then diffused into the serum. That the corpuscles are freely permeable to carbonic acid is now established, but the transfer of cations between the corpuscles and the plasma or serum seems very improbable.

In seeking an explanation for the increased titratable alkalinity of

[112] *Centr. med. Wiss.* (1867), 529; Dissertation, Bonn (1868).

the serum when carbon dioxide is passed through blood, Gürber [113] analyzed the ash of the serum and determined that no sodium or potassium diffused from the corpuscles, but that sufficient chloride passed into the corpuscles to account for the increase in serum bicarbonate.[114]

The exchange of bicarbonate for chloride ions and migration of the latter into or out of the red cell is described as the *chloride shift* (Fig. 29). The essential facts are that the red-cell membrane is permeable to water, carbon dioxide, and bicarbonate (HCO_3) and chloride ions, and that the cell-plasma ratio for chloride tends to become the same as that for bicarbonate. When carbon dioxide produced in the tissues enters the blood, a considerable part passes into the corpuscles where it is hydrated (through the action of the enzyme, carbonic anhydrase) and neutralized by the cell buffers. Entrance of HCO_3 into the corpuscle is accompanied by the outward passage of chloride; on the other hand, diffusion of the bicarbonate ion from cell to plasma and its accumulation therein involves a shift of chloride ions from the plasma into the corpuscles. That the transfer of chloride between plasma and corpuscles under the influence of changing tensions of carbon dioxide occurs *in vivo* has been demonstrated by numerous workers who compared arterial with venous blood and found more chloride in the plasma of the former than in that of the latter.

Electrolyte and Gas Equilibria. The distribution of electrolytes in the blood, including the transfer of chloride, hydrogen ions, carbon dioxide, and water between the plasma and corpuscles, may be explained partly on the basis of Donnan's theory of membrane equilibrium, by assuming that the membrane of the red cell is the semi-permeable membrane which separates the plasma from the fluid in the corpuscles. This membrane, as we have seen, is impermeable to proteins and cations, with the exception of H^+ ions. It is permeable to HCO_3^- and other anions (Cl^-, $SO_4^=$, and $PO_4^=$). Electrolytes that are present on either side of the membrane will tend to distribute themselves equally on the two sides. This tendency will be opposed, however, by the attractive forces of the non-diffusible ions, with the result that when equilibrium is reached there will be an uneven distribution of the diffusible ions on the two sides of the membrane.

The study of the blood as a physicochemical system by several groups of investigators in this country and abroad has materially advanced our knowledge of its properties. The relations involved in the distribution of gases and electrolytes between the cells and serum are very complex and do not lend themselves to a brief formulation. For this reason they cannot be further considered here, but if the student is to gain an appreciation of the scope of the subject he is urged to consult the mono-

[113] Maly's Jahresb., **25**, 165 (1895).

[114] Van Slyke (*Physiol. Rev.*, **1**, 161 [1921]) was able to account for only 72 per cent of the alkali increase of the plasma on the basis of the chloride shift. Other anions, SO_4 and PO_4, are also capable of migration through the red-cell membrane. See also Doisy, Eaton, and Chouke, *J. Biol. Chem.*, **47**, 377 (1921).

graphs of Van Slyke,[115] L. J. Henderson,[116] and Peters.[117] The composition of the cells and serum with respect to any given constituent, at any stage in the respiratory cycle, is quantitatively dependent on practically all the other constituents. In other words, each is variable, and the relations of all the variables may be mathematically formulated and graphically represented. An idea may be gained of the complexity of the situation if some of the variables are enumerated. These are:

1. Concentration of Cl in serum (Cl_s).
2. Concentration of Cl in cells (Cl_c).
3. The percentage of total blood chloride or bicarbonate present in the cells (A).
4. Volume of cells (V).
5. Percentage of H_2O in cells.
6. The base combined with cell protein (BP_c).
7. The combined carbonic acid of the cells ($[BHCO_3]_c$).
8. The combined carbonic acid of the serum ($[BHCO_3]_s$).
9. Total CO_2 of the blood.
10. Free CO_2 of the blood.
11. Base bound by protein of serum (BP_s).
12. pH in the serum.
13. pH in the cells.
14. Oxygen pressure.
15. Combined oxygen (HbO_2).

Further reference to the distribution of electrolyte between the cells and plasma will be made in considering the inorganic composition of the blood (p. 272).

Hydrogen-ion Concentration of the Blood. The blood is slightly alkaline in reaction, the serum being more alkaline than the corpuscles, arterial blood more than venous. This is shown by the following illustrative data obtained in two normal subjects at rest:

TABLE XXXIV

COMPARISON OF pH IN NORMAL RESTING INDIVIDUALS*

	I		II	
	Serum	Cells	Serum	Cells
Arterial	7 155	7 118	7 442	7 108
Venous	7 429	7 110	7.416	7 098
Difference	0 026	0 008	0 026	0 010

* After L. J. Henderson, "The Blood," Yale Univ. Press, 1928, pp. 195, 201. Somewhat different data have been reported more recently from Henderson's laboratory. Dill, Edwards, and Consolazio (*J. Biol. Chem.*, **118**, 635, 649 [1937]) found the arterio-venous difference to be 0 032 (pH 7.40 − 7.368) for serum and 0 023 (pH 7.190 − 7.167) for cells. At an altitude of 5.35 km. (20,000 feet) the corresponding value for serum was 7.376 − 7.352 = 0.024, and for cells 7.178 − 7.159 = 0.019.

[115] D. D. Van Slyke, "Factors Affecting the Distribution of Electrolytes, Water and Gases in the Animal Body," Lippincott, Philadelphia and London, 1926, p. 28.

[116] L. J. Henderson, "Blood, A Study in General Physiology," Yale Univ. Press, 1928.

[117] J. P. Peters, "Body Water, The Exchange of Fluids in Man," Chapter V, C. C. Thomas, Springfield and Baltimore, 1935.

As indicated by the foregoing data the difference in *p*H between arterial and venous blood is small, the cells being affected even less than the plasma.

The following normal variation in serum *p*H were reported by Shock and Hastings:[118]

Men: *p*H 7.35–7.45 (average 7.399; standard deviation of distribution, 0.064.

Women: *p*H 7.37–7.47 (average 7.415; standard deviation of distribution, 0.025).

A greater shift in *p*H between arterial and venous blood as well as increased acidity above that in the resting state occur as a result of work. This is illustrated by the following data:

TABLE XXXV

COMPARISON OF THE *p*H OF THE BLOOD OF A NORMAL MALE INDIVIDUAL, AT REST AND AT WORK*

	Rest		Work	
	Serum	Cells	Serum	Cells
Arterial	7 425	7 124	7 351	7 062
Venous	7 399	7 106	7 278	7 027
Difference	0 026	0 008	0 078	0 035

* Based on data given by L. J. Henderson, Chapter IX.

Even more conspicuous changes in the hydrogen-ion concentration of the blood may be produced by very strenuous exercise[119] or by rebreathing air through a closed system.[120] An increase in alkalinity of the blood may be produced physiologically by overbreathing. In this way, Davies, Haldane, and Kennaway[121] obtained values as high as *p*H 7.85. Ordinarily, any marked divergence from the normal is indicative of serious disturbance of the acid-base equilibrium of the body. A value higher than *p*H 7.5 represents a condition of *alkalosis*. It may be due, as will be shown presently, either to an uncompensated excess of alkali or to an uncompensated deficit of carbon dioxide. On the

[118] *J. Biol. Chem.*, **104**, 565 (1934).

[119] Barr, Himwich, and Green have reported a value as low as 7.05 (*J. Biol. Chem.*, **55**, 495 [1923]). Compare these observations with those of H. A. Rice, and A. H. Steinhaus, *Am. J. Physiol.*, **96**, 529 (1931).

[120] When air is rebreathed, its CO_2 tension increases. The effect on the *p*H of the blood is a marked reduction. In one subject studied by Shock and Hastings (*J. Biol. Chem.*, **112**, 239 [1935]), the *p*H of the serum diminished to 7.19 in 20 minutes.

[121] *J. Physiol.*, **54**, 32 (1920).

other hand, a *p*H below 7.3 indicates a condition of *acidosis*, due either to an uncompensated deficit of alkali or to an uncompensated excess of carbon dioxide. Values lower than *p*H 7.0 are rarely encountered in individuals who later recover. Such values are indicative of extreme acidosis and have been observed almost exclusively in cases of diabetic or uremic coma which terminated fatally.

Acid-base Balance. *Alkali Reserve.* The alkali reserve refers to the amount of base combined as bicarbonate and not to all the base stored in the blood, as is sometimes supposed.[122] Bicarbonates afford a readily available source of base for the neutralization of acids stronger than carbonic acid. Base present in excess of acids other than carbonic takes the form of bicarbonate. The designation of this source of base as " alkali reserve " receives added justification in the fact that it reflects more or less closely the reserve of available alkali present in the body as a whole.

According to Peters and Van Slyke,[123] " *acidosis* may be broadly defined as an abnormal condition caused by the accumulation in the body of an excess of acid or the loss from the body of alkali." Acids tend to accumulate when they are formed or absorbed more rapidly than they are destroyed or eliminated. When this process has caused either the bicarbonate of the blood to fall (decreased alkali reserve), or the hydrogen-ion concentration to rise above the normal limits, a state of acidosis is said to exist.

Similarly, *alkalosis* may be defined as an abnormal condition caused by the accumulation in the body of an excess of alkali (increased alkali reserve), or by the loss of acid. Either the accumulation of alkali or the loss of acid exerts the same effect, namely to increase the bicarbonate and usually to diminish the hydrogen-ion concentration of the blood.

We shall consider in particular the relation between carbonic acid and base in the blood, inasmuch as this constitutes the principal factor in the acid-base equilibrium. As ordinarily employed the term blood *p*H actually refers to the *p*H of venous blood, plasma, or serum. As has been stated elsewhere, the normal *p*H is approximately 7.4, corresponding to an hydrogen-ion concentration of 4×10^{-8}. For the extreme normal range, the values *p*H 7.3 to 7.5 are usually cited; however, the values 7.35 to 7.47 obtained by Shock and Hastings for the two sexes are probably more nearly correct. Correspondingly, the carbon

[122] This is evident from the methods used in its determination, one of the simplest being the method introduced by Van Slyke and Cullen in 1917. This procedure is based upon the assumption that the CO_2-combining power of the blood depends upon the amount of alkali which is available. The essential features of the method consist in saturating the blood, after it is drawn, with carbon dioxide, and liberating the CO_2 in a definite amount of blood by treating it with acid *in vacuo* in an apparatus devised for this purpose. *J. Biol. Chem.*, **30**, 289 (1917). This method has been modified so that the CO_2 liberated is measured manometrically (*ibid.*, **61**, 523, 575 [1924]).

[123] "Quantitative Clinical Chemistry," p. 870.

dioxide content varied between 22-30 mM. per liter (49–67 volumes per cent), and the carbon dioxide tension from 36.5 to 50 mm. of mercury.

Deviation from the normal acid-base balance is of two general types:

(1) Metabolic, due primarily to (*a*) alkali excess, (*b*) alkali deficit.
(2) Respiratory, due primarily to (*a*) CO_2 excess, (*b*) CO_2 deficit.

Primary alkali excess may be due either to the retention of alkali or the loss of acid. The administration of alkaline substances, such as sodium bicarbonate, in sufficient amount, results in an alkali excess. The condition is characterized by an increased CO_2 content due primarily to the rise in bicarbonate. Corresponding to this is a rise in *p*H. which if sufficient may be accompanied by the development of tetany. Values as high as *p*H 7.8 have been encountered, not infrequently.

Copious vomiting of HCl, as in pyloric obstruction, may cause a *relative* excess of alkali and a corresponding rise in *p*H.

In an earlier classification, Van Slyke described primary alkali excess that was not balanced by a change in CO_2 tension as *uncompensated* alkali excess. As the primary alkali change is followed by a corresponding increase in CO_2, or by the excretion of alkali, the ratio $\frac{HHCO_3}{BHCO_3}$ tends to return to normal, as does also the *p*H of the blood. When the normal ratio is thus restored, even though the content of base remain high, the condition is one of *compensated* alkali excess.

Primary alkali deficit represents a condition in which the most conspicuous and constant change is the reduction of the bicarbonate (and CO_2) concentration. When the reduction in bicarbonate is not balanced by a corresponding change in carbonic acid, the *p*H diminishes. The condition may result from (*a*) acid retention, (*b*) alkali loss. In most instances acid formation occurs first, followed by neutralization and the excretion of the salts thus formed. In consequence there is a loss of base from the body. The two factors, namely, acid formation and alkali depletion, are frequently coexistent.

In diabetes, as the result of faulty fat metabolism, large amounts of acetoacetic acid and β-hydroxybutyric acid are formed. These are eliminated in combination with base, thus depleting the alkali reserve of the body. The same factors are involved in fasting and carbohydrate deprivation (p. 361).

The immediate effect of the administration of acids, or acid-forming salts, such as NH_4Cl, is the retention of acid. NH_4Cl is converted into urea and HCl. Neutralization of the latter involves a loss of available base and hence a reduction of serum bicarbonate. The *p*H is lowered.

Retention of NaH_2PO_4 through the failure of the kidneys to excrete it, as in terminal nephritis, causes a relative increase of the ratio $\frac{NaH_2PO_4}{Na_2HPO_4}$, as well as a similar change with respect to other buffer pairs.

There is an accompanying reduction in the *p*H. The end result is a relative alkali deficit.

During pregnancy the total serum base is somewhat diminished, but this is compensated by a comparable reduction in H_2CO_3, so that the *p*H tends to remain within normal limits.

Primary CO_2 excess is a condition characterized by a low *p*H and a high or normal CO_2. It occurs when the excretion of CO_2 is retarded, usually as a result of some obstruction to the passage of air to and from the lungs. Though relatively uncommon, this condition has also been observed in cases of cardiac decompensation accompanied by dyspnea, and in morphine narcosis. In essential emphysema a condition of CO_2 excess may develop, but it is usually compensated. Scott[124] has recorded one such instance with a CO_2 content of 82.7 volumes per cent and with a normal *p*H of 7.4.

Primary CO_2 deficit is a condition of abnormally high *p*H (7.6 to 7.8) with a normal or low CO_2 content. The latter may fall to as low a level as 20 to 30 volumes per cent. The condition may result from voluntary or involuntary overventilation of the lungs.[125] If the degree of alkalosis is sufficient, symptoms of tetany may develop. Primary CO_2 deficit is also observed at high altitudes (or in atmospheres with low oxygen tension) and may likewise be associated with an increase in body temperature, due to hot baths, fever, etc. The disturbance in acid-base balance occurring under all these circumstances is gradually modified by a diminution in alkali reserve (through the excretion of base), corresponding more or less to the reduction in H_2CO_3, and a consequent restoration to the normal *p*H range ("compensated CO_2 deficit").

In a series of experiments on normal subjects, Shock and Hastings [126] produced displacement of the acid-base equilibrium in a variety of ways (feeding sodium bicarbonate or ammonium chloride, rebreathing, overbreathing). Among their observations, one is of particular interest as it suggests that individuals may be characterized in terms of the rate at which the acid-base balance of the blood is restored to normal after experimental displacement.

COMPOSITION OF THE BLOOD

Hemoglobin. Normally the amount of hemoglobin varies with age and sex. The highest values are encountered during the first 2 to

[124] *Arch. Internal Med.*, **26**, 544 (1920).

[125] H. W. Davies, J. B. S. Haldane, and E. Kennaway, *J. Physiol.*, **54**, 32 (1920–21).

[126] *J. Biol. Chem.*, **112**, 239 (1935). Shock and Hastings refer to the condition of *CO_2 deficit*, such as results from overventilation, as *respiratory alkalosis*, while the condition of *CO_2 excess* is described as *respiratory acidosis*. *Acid excess*, whether due to the ingestion of acid, abnormal production in metabolism, loss of fixed base, or failure in excretion, is designated as *metabolic acidosis*, whereas the condition of *alkali excess*, due to loss of fixed acid, or ingestion of base, is termed *metabolic alkalosis*.

3 days of life (22 to 23 grams per 100 cc. of blood, corresponding to an oxygen capacity of 27 to 29 volumes per cent).[127] Available data indicate a gradual diminution to about 13 grams (oxygen capacity of 16 volumes per cent), followed after the fourth or fifth year by a gradual rise to the adult values, attained at about the age of 16 years. For males, the average adult level is 16 grams per 100 cc. (oxygen capacity of about 20 volumes per cent); for females the average is about 14 grams (oxygen capacity of 18 volumes per cent).

Physiologically the hemoglobin content increases at high altitudes, and as a result of vigorous exercise, largely because of a corresponding change in the concentration of the blood (diminished plasma volume), particularly in the latter case. Diurnal variations and changes associated with digestion, water administration, dehydration, pregnancy, and emotional, seasonal, climatic, and other factors have been described.

Pathologically the amount of hemoglobin is reduced in various forms of anemia.

Inorganic Constituents. Sodium, potassium, calcium, and magnesium are the quantitatively important basic constituents of the tissues and body fluids and exist in combination with the following inorganic and organic anions: HCO_3', Cl', PO_4''', SO_4'', protein, lactic acid, and other organic acid radicals. Of these the monovalent ions, $K^{\cdot}$, $Na^{\cdot}$, Cl', HCO_3', and monovalent organic acid anions (lactic acid, etc.), compose about 95 per cent of the total electrolytes of the blood plasma and other extracellular fluids. Their approximate distribution in human blood is represented by the following data:

TABLE XXXVI

	Serum Mg. per 100 cc.	Cells Mg. per 100 cc.
$Na^{\cdot}$	335 ± 15	0
$K^{\cdot}$	19 ± 3	420 ±
$Ca^{\cdot\cdot}$	10 ± 1	0 (0 53)
$Mg^{\cdot\cdot}$	2 74 ± 0 3	6 61 ± 0 53
HCO_3'	164 ±	112 ±
Cl'	370 ± 20	190 ± 20
PO_4'''	10 ±	18 ±
SO_4''	19	—

[127] Theoretically, 1 gram of hemoglobin when fully saturated should combine with 1.34 cc. of oxygen. There are, however, no reliable data of the maximum volume of oxygen capable of combining with hemoglobin (Van Slyke and Peters, "Quantitative Clinical Chemistry," Vol. 1, p. 524). In arterial blood, oxygen is normally about 95 per cent saturated.

A better way of expressing these concentrations is in terms of milliequivalents,[128] as follows:

TABLE XXXVII

BASES IN SERUM AND CELLS*

	Concentrations in Milliequivalents per Liter	
	Serum	Cells
Na.....	135 1	16.8
K.....	4 6	82.5
Ca	5 3	0.2
Mg	1.6	4 6
Total base (B).	146.6	104.0
Total base per kilo of H_2O† . . .	156 4	146 4

* Average results obtained by P. M Hald and A J Eisenman, *J. Biol Chem*, **118**, 275 (1937).

† Owing to the disparity in the proportion of solids in cells and serum, the concentrations in terms of a unit of water (1 liter) are obviously of more significance than other modes of expression, especially from the standpoint of evaluating their osmotic effect.

It may be supposed that the total concentration of osmotically active ions would be represented by twice the concentration of base, or 2B.

[128] In the form in which the quantitative relation of these constituents is expressed in Table XXXVI, it is not apparent that a balance exists between the acid and basic radicals. Indeed the conventional method of expressing the results of quantitative analysis in terms of proportion by weight is unsatisfactory, especially in certain cases where it is desirable to bring out the relations of the various constituents to one another.

A better method is to express the amounts in terms of their equivalence. The significance of the term *molar* (*molal*) and *normal*, as applied to solutions, is probably familiar to the student. A *normal* solution of acid contains 1.008 grams of replaceable hydrogen per liter. This is approximately equivalent to 35.5 grams of reactive chloride, 23 grams of sodium, or 20.04 grams of calcium. Ordinarily, the various constituents of the body fluids are present in small concentration and it is therefore more convenient to express the results in *millimoles* (one-thousandth of a mole per liter) or still better in milliequivalents (*m. eq.*). A few simple examples will illustrate the point:

Serum contains 335 mg. of sodium per 100 cc. This is equivalent to 3350 mg. per liter. Inasmuch as 1 *m. eq.* of sodium weighs 23 mg., the concentration is 3350/23 = 146 *m. eq.* per liter.

The concentration of chloride is 360 mg. per 100 cc. of serum. This corresponds to 3600/35.5 = 101 *m. eq.* per liter.

The value for serum calcium is 10.6 mg. per 100 cc. The atomic weight of Ca is 40.08; 1 *m. eq.* therefore weighs 20.04 mg. Hence $10.6 \times 10/20.04 = 53$ *m. eq.* per liter.

For certain purposes it is desirable to express the concentrations in terms of milliequivalents per 1000 cc. of water. Thus, if the chloride concentration of serum is 101 *m. eq.* and the content of water 92 per cent, $101/92 \times 100 = 109.8$ *m. eq.*, is the chloride content of 1000 cc. of serum H_2O.

However, this assumption is evidently incorrect, for Peters [129] and others have shown that there is a marked discrepancy in the concentrations of total base and total acid (normal human blood).

	M. eq. in 1000 cc. of Water	
	Serum	Cells
Total base.....	155	144
Total acid.....	160	189

The small surplus of acid ions in serum is apparently related to the existence therein of an unionizable lipoid-chloride complex which may be extracted with petroleum ether. The much greater divergence in the cells has not been entirely accounted for, but it seems probable that both CO_2 and Cl' are present in combination with organic cellular constituents, such as protein. The reaction within the cell is such that protein may hold these ions in unionizable combination. The occurrence of an hemoglobin-carbamino compound (p. 257) strengthens this supposition.

Sodium and Potassium. In the blood of man, sodium predominates in the plasma and potassium in the corpuscles. This is also true for the rabbit, pig, rat, guinea pig, and monkey, but not for the cat, dog, sheep, or cow. In the blood of the latter group, sodium predominates in the corpuscles as well as in the plasma.[130]

A diminished alkali reserve, from whatever cause, is accompanied by a definite reduction of the sodium and potassium concentrations of the blood. Profuse sweating, or diarrhea, may produce a similar effect despite the marked dehydration which may coexist. In adrenal insufficiency, the concentration of sodium in the serum is conspicuously diminished. The potassium and calcium tend to rise above normal.

Sodium is the predominant base in lymph, edema fluid, cerebrospinal, and other extracellular fluids. Potassium predominates in muscle. The problem of salt and water exchange between muscle and blood has been a recent subject of investigation by Hastings and Eichelberger.[131]

Calcium. Blood serum normally contains from 9 to 11.5 mg. of calcium per 100 cc. Rymer and Lewis [132] found on an average only

[129] J. P. Peters, "Body Water," p. 99.

Peters' data for the distribution of acid ions and groups (those capable of combining with base) are as follows (in milliequivalents per 1000 cc. of water):

	Protein*	Bi-carbonate	Chloride	Organic phosphate	Inorganic phosphate	Sulfate	Total
Serum..	17	28	111	...	3	1	160
Cells....	62	27	74	23	3	..	189

* This represents the base-combining value; estimates of the actual concentration are: 2 m. eq. for serum and 7 m. eq. for cells.

[130] For recently published data on the subject, see H. Yannet, D. C. Darrow, and M. K. Cary, *J. Biol. Chem.*, **112**, 477 (1936), and S. E. Kerr, *ibid.*, **117**, 227 (1937).

[131] *Ibid.*, **117**, 73; **118**, 197, 205 (1937).

[132] *J. Biol. Chem.*, **95**, 441 (1932).

0.53 mg. of calcium per 100 cc. of corpuscles, and others have reported entirely negative results. Apparently the red-cell membrane is impermeable to calcium ions.

Of the serum calcium about half (4.5–5.5 mg. per 100 cc.) is diffusible through a collodion membrane and exists in a readily ionizable form. The non-diffusible calcium is combined with protein. Cerebrospinal fluid, which may be regarded as an ultrafiltrate of blood plasma, contains about 5 mg. of calcium per 100 cc., practically all of which is in an ionized and diffusible form. [133]

The constancy of the serum calcium concentration is very striking and reflects the proper balance of various forces. If the calcium intake is adequate to meet all requirements and if absorption and utilization are assured through the presence in the diet of vitamin D (p. 604), the normal level is maintained, provided no abnormality of parathyroid function exists. In conditions of parathyroid deficiency, such as may result from atrophy of the glands, or their extirpation, the serum calcium is markedly diminished. When the concentration falls below 7 mg. per 100 cc., tetany often develops. The onset of convulsions probably depends not so much on the reduction of the total calcium as on the fall of the ionizable fraction. If the latter is diminished below 3.5 mg. per 100 cc., clinical symptoms of tetany are likely to develop. This effect of hypocalcemia may be overcome, at least temporarily, by the administration of calcium salts. Milk has also been reported to exert a beneficial effect.

Tetany associated with rickets and osteomalacia is due to hypocalcemia resulting from nutritional deficiency in respect to calcium (see p. 543).

The serum calcium concentration tends to diminish in the last months of pregnancy, often below 9 mg. per 100 cc. This is due, at least in part, to the increased demands of the growing fetus. If the

[133] There has been a difference of opinion concerning the state of the calcium in body fluids. One group has held that only part of the diffusible calcium (about 2 mg. per 100 cc.) is ionized, the remainder being bound in some organic combination, such as citrate. Others have advanced the view that all, or nearly all, of the diffusible calcium is in ionic form. The lack of appropriate methods for determining calcium ion has delayed a direct attack on the problem. However, McLean and Hastings have devised an ingenious biological method of assay, based on the extraordinary sensitivity of the isolated frog's heart to changes in the calcium content of the nutrient fluid. Their studies of normal and pathological bloods and of cerebrospinal and other body fluids has led them to conclude that probably all the diffusible calcium is in ionized form and that, if there is a diffusible, non-ionized compound or fraction, it certainly does not represent more than 5 per cent of the total diffusible calcium. For normal serum the calcium-phosphate ion product is: $(Ca^{++}) \times (PO^{\equiv}_4) = 10^{-12.5}$. The calcium-carbonate ion product is $(Ca^{++}) \times (CO^{=}_3) = 10^{-6.7}$ (Logan and Taylor, Hastings). References: I. Greenwald, *J. Biol. Chem.*, **67**, 1 (1926); D. M. Greenberg and C. E. Larson, *ibid.*, **109**, 105 (1935); H. R. Benjamin, *ibid.*, **109**, 123 (1935); F. C. McLean and A. B. Hastings, *Am. J. Med. Sci.*, **189**, 601 (1935); *J. Biol. Chem.*, **107**, 337 (1934); **108**, 285 (1935); M. A. Logan and H. L. Taylor, *ibid.*, **119**, 293 (1937); A. B. Hastings, *New Eng. J. Med.*, **216**, 377 (1937).

combined maternal and fetal requirements are not met, the calcium reserves of the maternal organism (bones, teeth) are drawn on. Part of the serum calcium reduction may be associated with a lowered protein concentration. In various other conditions (nephritis, nephrosis, malignancy, liver disease, etc.) in which there is serum protein deficiency, the serum calcium is subnormal.

That there is a relationship between serum protein and serum calcium has been stressed by many investigators. According to Darrow and Cary [134] it is expressed by the following equation:

$$\text{Ca} = 0.47 \times \text{protein} + 2.89$$

Ca is expressed in milliequivalents per liter; protein, in percentage.

Magnesium. Greenberg and associates [135] found the average magnesium content of human serum to be 2.74 mg. per 100 cc. In 70 per cent of their 58 subjects, the values fell between 2.45 and 3.05 mg. The extreme range observed was 2 to 3.6 mg. The magnesium content of the red blood corpuscles averaged 6.61 mg., with a standard deviation of $\pm$ 0.53 mg. The cell membrane appears to be relatively impermeable to this ion. It is considered probable that magnesium may play some special physiological rôle in the corpuscle where it is the sole representative of the alkaline-earth elements, the calcium content being negligible.

The magnesium level is practically unaffected by the concentrations of serum protein or phosphate. Very little is known of its variations physiologically and in disease.

Chloride and Bicarbonate. The anions Cl^- and HCO_3^- may be considered jointly because of their interdependence. Factors which alter the concentration of one tend to produce a reciprocal change in the other. This is indicated by the data in Table XXXVIII.

The concentration of chloride in normal human serum may vary from about 350 to 380 mg. per 100 cc. (99 to 108 m.eq. per liter) corresponding to 0.56 to 0.63 per cent of sodium chloride. Ordinarily, however, the range of values is somewhat narrower, 362 to 376 mg. Cl^-, or 596 to 620 mg. NaCl, per 100 cc. (102 to 106 m. eq. per liter).

On a salt-free diet, the Cl^- concentration is lowered to about 98 mM., at which "threshold" level it remains fairly constant, inasmuch as little or no chloride is excreted in the urine under the circumstances. In contrast, if large amounts of salt are administered (40 g. daily, or more), the serum Cl^- concentration may be raised to 110 mM., but above this point it is difficult to cause a further increase in the normal individual, because of the rapid excretion of the excess by the kidneys.

[134] *J. Biol. Chem.*, **105**, 327 (1934); see also I. Greenwald, *ibid.*, **93**, 551 (1931); A. B. Gutman and E. B. Gutman, *J. Clin. Investigation*, **16**, 903 (1937).

[135] *J. Biol. Chem.*, **100**, 139 (1933); for a comparison of the distribution of magnesium in blood cells and plasma of rat, guinea pig, rabbit, chicken, horse, swine, goat, sheep, and cattle, see D. F. Eveleth, *ibid.*, **119**, 289 (1937).

The intravenous administration of hypertonic salt solution causes a temporary increase in concentration, followed rapidly by the withdrawal of water from the tissues and an increase in blood volume.

TABLE XXXVIII

RELATION OF Cl^- AND HCO_3^- IN ARTERIAL AND VENOUS BLOOD, AT REST AND AT WORK*

	Subject I at Rest		Subject II at Rest		Subject I at Work	
	Arterial	Venous	Arterial	Venous	Arterial	Venous
Cl^-, mM. per liter serum	99 32	98.49	102 56	102 03	102.7	100 3
Total CO_2 (HCO_3^-) mM. per liter serum	26 57	28 50	27.16	28 79	20 86	25.84
Cl^-, mM. per liter cells	45 27	47 00	47 03	48 27	49 42	53.22
Total CO_2 (HCO_3^-) mM. per liter cells	13 98	15 40	15 59	17 07	13.08	17.03

* After L. J. Henderson, "Blood," Yale Univ Press, New Haven, 1928.

During the early stages of digestion, associated with gastric activity and the secretion of HCl, the serum chloride diminishes. This is accompanied by a compensatory increase in bicarbonate. The reverse occurs during anesthesia, namely an increase in Cl^- and a decrease in HCO_3^- ions. As a result of the administration of bicarbonate, a reduction in plasma chloride occurs.

Subnormal chloride values are observed during starvation, but the most striking change is caused by the vomiting in obstruction to the gastro-intestinal tract. A 50 per cent reduction of the serum chloride concentration is not an uncommon finding. Though a compensatory increase in bicarbonate occurs, it is usually less than the fall in chloride. The chloride concentration is also reduced in diabetic acidosis, acute glomerular nephritis, emphysema, and pneumonia. In patients with Addison's disease, in crisis, as well as in animals with experimental adrenal insufficiency, the chloride (and NaCl) is conspicuously depressed, the reduction being proportionately greater than the loss of sodium. There is a tendency to acidosis, as shown by diminution of the CO_2-combining power of the blood.

Low blood chlorides have been observed in association with heat cramps. Individuals working at high temperatures often lose considerable quantities of both salt and water. Failure to restore the salt may result in very dramatic symptoms (p. 288).

Phosphate. Youngburg and Youngburg [136] in a group of 12 normal subjects found the serum inorganic phosphorus to vary between 2.56

[136] *J. Lab. Clin. Med.*, **16**, 253 (1930).

and 4.43 mg. per 100 cc. (average 3.73), whereas the content in the corpuscles varied between 1.08 and 4.69 mg. (average 3.32).

An increase occurs in terminal nephritis. In rickets and osteomalacia, the phosphorus is low,[137] if the condition is primarily due to phosphorus deficiency. Physiologically a slight fall accompanies the active utilization of glucose. In the diabetic, depending on the capacity to metabolize carbohydrate, this fall is not so marked, and the return to normal is longer delayed than in the normal individual.

The inorganic (orthophosphate) phosphorus constitutes but a small fraction of the total. Various organic phosphorus compounds are present, chiefly in the corpuscles. The organic phosphorus compounds of the blood have been subdivided into two main fractions:

(*a*) Acid soluble, including adenosine triphosphate,[138] nucleotides, phosphoric acid esters, and other, undetermined compounds.

(*b*) Alcohol-ether soluble, or lipoid phosphorus.

Nucleic acid is not a constituent of the normal erythrocyte. Reticulated cells may, however, contain it, according to Kay.[139]

Further subdivision into fractions, such as enzyme-hydrolyzable, enzyme-non-hydrolyzable, etc., has been attempted, but on the whole our present knowledge of the constituents composing these fractions is fragmentary.

Such data as are to be found in the literature indicate the normal total phosphorus of the serum to be 8 to 18 mg., and of the corpuscles, 47 to 114 mg.

Sulfate. Blood contains 0.5 to 1.0 mg. per 100 cc. of inorganic sulfate sulfur. The amount of ethereal sulfate is approximately the same. About 2 to 4 mg. of sulfur is in the unoxidized, or so-called neutral, form and is present somewhat more abundantly in the cells than in the plasma. Increased concentrations have been reported in pyloric and intestinal obstruction, diabetes, and leukemia, but the most striking retention has been observed in patients with terminal nephritis.

Organic Constituents. The proteins of the plasma having been considered elsewhere, we shall restrict our attention to the (*a*) nonprotein nitrogenous constituents, (*b*) glucose, (*c*) lipids. Removal of the protein by suitable precipitants, under appropriate conditions, leaves in solution, besides inorganic salts, glucose, and a group of nitrogen-containing substances. The last are determined by analyzing the protein-free filtrate for total nitrogen; hence the term non-protein

[137] Bakwin, O. Bodansky, and Turner obtained an average of 5.41 mg. of inorganic (orthophosphate) P per 100 cc. of serum in non-rachitic children (36 subjects). The concentration in the cells averaged 2.48 mg. In contrast, values of 2.95 mg. for serum and 1.05 for cells were obtained in a group of 19 infants with rickets. *Proc. Soc. Exptl. Biol. Med.*, **29**, 1238 (1931–32).

[138] Kerr and Daoud have reported the results of a comprehensive study of the organic acid-soluble phosphorus of the erythrocyte of a number of vertebrates, *J. Biol. Chem.*, **109**, 301 (1935).

[139] *Brit. J. Exptl. Pathol.*, **11**, 148 (1930).

nitrogen (or total non-protein nitrogen, or N.P.N.). It represents the nitrogen of urea, uric acid, ammonia, the amino acids, creatine and creatinine, and certain undetermined substances (peptides, nucleotides, etc.). It is customary to record the concentration of at least some of these constituents, for example, urea, in terms of milligrams of nitrogen per 100 cc. of serum, plasma, or blood, as the case may be. Creatine, creatinine, and uric acid are usually expressed as such.

Non-protein Nitrogen. For whole blood the normal variation is from 25 to 35 mg., with an average of about 30 mg. Plasma contains 18 to 30 mg. (average about 25 mg.). Folin and Svedberg [140] analyzed the blood of 19 normal individuals and obtained the following data for non-protein nitrogen: [141]

TABLE XXXIX

Time	Plasma			Laked Blood			Unlaked Blood		
	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average
Before breakfast . . .	25 0	19 1	22 6	35.3	25 6	30 7	20 8	13 8	18 5
2½ hours after breakfast.	28 3	20 2	24 2	42 0	26 4	30 8	20 2	14 6	18 0

One factor influencing the level of non-protein nitrogen is the rate of protein metabolism. On a restricted protein intake, provided the diet is adequate otherwise, the blood N.P.N. may be reduced to approximately half the normal value. In contrast, when protein metabolism is increased, as in starvation, fever, and untreated severe diabetes, the N.P.N. tends to rise above normal.

The rate of excretion of nitrogen by the kidney is even more important in determining the level of N.P.N. Diuresis tends to lower it. Diminished urinary flow, as occurs in heart failure or as the result of dehydration accompanying severe diarrhea, persistent vomiting, fever, etc., tends to raise it. The increased N.P.N. observed in intestinal obstruction and in fever may also be due to impaired renal function. Obstruction to the urinary passages (stricture of the ureter or urethra, enlarged prostate, etc.) may be accompanied by a marked rise in N.P.N., values of 100 mg. or more being encountered not infrequently. As the

[140] Most of the data on the distribution of the various blood constituents have been obtained by the method of Folin and Wu, which involves preliminary laking of the blood. More recently Folin has pointed out that certain errors are inherent in the method. In the process of laking, unknown products presumably are formed or liberated from the disintegrated cells and constitute the major part of the undetermined nitrogen fraction. *J. Biol. Chem.*, **38**, 81 (1919); Folin, *ibid.*, **86**, 173 (1930); Folin and A. Svedberg, *ibid.*, **88**, 715 (1930).

[141] Compare the results in Table XXXIX with those of Wu, *J. Biol. Chem.*, **51**, 21 (1922).

obstruction is relieved, the blood N.P.N. tends to return to normal. The most conspicuous changes are observed, however, in disease of the kidney. In non-hemorrhagic (degenerative) Bright's disease renal function may remain fairly normal and the N.P.N. essentially unchanged, unless the urine output is markedly decreased. Even in acute glomerular nephritis, the ability to excrete the nitrogenous constituents may remain unimpaired, and hence no change occurs in the blood. More often, however, this is not the case; nitrogen retention develops, and, as in chronic nephritis, there is a rise in N.P.N. that is more or less commensurate with the degree of renal insufficiency. When the capacity of the kidney to concentrate is reduced to an extremely low point, as in the terminal stages of Bright's disease, the non-protein nitrogen rapidly attains very high values, 300 mg. or even more.

Amino-acid Nitrogen. The amino acids of the blood are to be regarded as nutrient material *en route* to the tissues. After a protein-rich meal, there is an increase in the amino-acid nitrogen of the blood.

In a series of 20 analyses of the blood of normal individuals, Greene, Sandiford, and Ross [142] found the amino-acid nitrogen to vary between 5.2 and 7.2 mg. per 100 cc. Approximately the same results were obtained in a series of more than 400 observations on individuals suffering from various pathological conditions, the concentrations varying between 4.8 and 7.8 mg. per 100 cc. of whole blood. The concentration of amino acids is greater in the corpuscles than in the plasma, according to Wu, [141] who obtained an average of 5.52 mg. per 100 cc. of plasma and 9.47 mg. per 100 cc. of corpuscles. However, data indicating that diffusible amino acids are more abundant in the plasma than in the corpuscles have been published by Folin and Svedberg.[140] Danielson,[143] working in Folin's laboratory, has concluded that the values based on analysis of laked blood are too high. Analyzing unlaked blood, he obtained the following data for the amino nitrogen in normal *fasting* individuals:

TABLE XI.

	Whole Blood		Plasma		Corpuscles	
	Average	Range	Average	Range	Average	Range
Young men (29 analyses) ..	3 0	2 3 –3.73	4 84	4 0 –5 65	1 04	0 34–2.19
Young women (8 analyses) .	2 89	2 32–3 3	4 78	3 86–5 46	0 37	0.05–0 834

[142] *J. Biol. Chem.*, **58**, 845 (1923-24); see also Blau, *ibid.*, **56**, 861 (1923).

[143] *Ibid.*, **101**, 523 (1933). Compare D. D. Van Slyke and E. Kirk, *ibid.*, **102**, 651 (1933).

A marked increase in amino-acid nitrogen and a corresponding reduction in urea nitrogen occur in acute yellow atrophy. It is only in the most severe forms of liver disease that destruction of the tissue is sufficient to interfere with the function of the liver in the metabolism of amino acids and in the production of urea (p. 412).

Urea. The concentration of urea in the blood varies within rather wide limits, as indicated by the data in Table XLI, but as a rule it represents about 50 to 60 per cent of the total non-protein nitrogen.

TABLE XLI

UREA NITROGEN IN MILLIGRAMS PER 100 CC.

Authority	Whole Blood			Plasma		
	Max.	Min.	Aver.	Max.	Min.	Aver.
Wu [141] (20 subjects)	22	12	17.1	23	13	19.3
Folin and Svedberg [140] (19 subjects)	14.5	9.8	13	16	10 5	13.8
MacKay and MacKay [144] 114 male subjects	21.6	12 05	15 4			
47 female subjects	18 2	5 14	11 4			

The data of MacKay and MacKay show that the average for the male group is about 35 per cent above that for the female group.

Urea is about equally distributed between the water of the corpuscles and of the plasma.

The concentration of blood urea is influenced by (1) the amount of protein metabolism and (2) the rate of excretion. It tends to be high when protein metabolism is increased by diet, fever, etc., and is somewhat diminished when protein metabolism is at a low level. With impairment in renal function and retention of nitrogen, the urea accumulates in greater proportion than the rest of the nitrogenous constituents, so that the ratio of $\frac{\text{Urea N}}{\text{N P N}} \times 100$ increases above 50 to 60.

Normally the concentration of blood urea is related to the excretion. This relation has been utilized clinically in estimating the functional activity of the kidneys (p. 459).

As has been stated, blood urea is markedly diminished in acute yellow atrophy. Aside from kidney disease, blood urea is markedly increased in Addison's disease, as well as in experimental adrenal-cortical insufficiency.

Uric Acid. The normal variations are represented by the following data:

[144] *J. Clin. Investigation*, **4**, 127 (1927).

TABLE XLII

BLOOD URIC ACID IN MILLIGRAMS PER 100 CC.

	Whole Blood			Plasma			Corpuscles		
	Max.	Min.	Aver.	Max.	Min.	Aver.	Max.	Min.	Aver.
Wu[141] (20 subjects).	.		. . .	5 7	2 3	3 92	3 8	1 2	1.93
Folin and Svedberg[140]	3 2	2 2	2 6	3 9	3 3				
Benedict and Behre[145]....	5 2	3 0	3 8	5 5	3 4	4.4	. . .	. . . .	3 17

In the new-born infant, during starvation, after exercise, during pregnancy, and especially at the end of labor the uric acid content of the blood is increased above normal. High uric acid values, due either to increased nuclear metabolism or retention, have been observed in various diseases, notably leukemia, polycythemia, arteriosclerosis, gout, toxemias of pregnancy, especially eclampsia of the nephritic type, and nephritis. In the terminal stages of nephritis it is not unusual to find the uric acid in the vicinity of 20 mg. per 100 cc., or even higher.

Creatine and Creatinine. Blood of normal individuals contains 1.0 to 1.5 mg. of creatinine per 100 cc., the distribution between the plasma and corpuscles being about equal.[146]

The concentration of creatinine is relatively constant and unlike other nitrogenous blood constituents is not subject to physiological variation. Even in nephritis, the creatinine level may remain unchanged

[145] *J. Biol. Chem.*, **92**, 161 (1931).

[146] The determination of creatinine depends on a reaction with alkaline picrate (Jaffé's reaction). As this is a non-specific reaction, various authorities have questioned the existence of creatinine, as such, in the blood (Behre and Benedict, *J. Biol. Chem.*, **52**, 11 (1922); **110**, 245 (1935); **117**, 415 (1937). Gaebler has reported that a part of the chromogenic material of serum ultrafiltrate resembles creatinine in its precipitation reactions (picrate, rubidium chloride), but has not succeeded thus far in demonstrating that the two are identical (*J. Biol. Chem.*, **117**, 397 [1937]); see also *ibid.*, **76**, 337 (1928), **89**, 451 (1930). See also J. M. Hayman, S. M. Johnston, and J. A. Bender, *ibid.*, **108**, 675 (1935); I. S. Danielson, *ibid.*, **113**, 181 (1936). Goudsmit, *ibid.*, **115**, 613 (1936), compared the apparent creatinine content of renal venous and arterial blood and found the concentration consistently lower in the venous blood. Although the difference is of a sufficient order of magnitude to consider the substance removed by the kidney as being probably creatinine, Goudsmit avoids this conclusion. See, however, M. K. Zacherl, *Z. physiol. Chem.*, **248**, 80 (1937). Miller and Dubos, *J. Biol. Chem.*, **121**, 429 (1937), have recently shown that an enzyme decomposing creatinine also destroys almost all the chromogenic material in normal plasma and about half of the quantity found in erythrocytes. This seems to be incontrovertible evidence that the blood constituent which has long been considered to be creatinine is, in fact, that compound.

so long as renal function is only moderately impaired. But when marked retention occurs, the creatinine tends to rise. Values above 2 mg. are definitely abnormal; 3 mg. indicates severe impairment in function, and 5 mg. is considered by clinicians of grave significance. In a case of bichloride of mercury poisoning in which there was almost complete anuria for 10 days, a creatinine value of 19.2 mg. per 100 cc. has been recorded (Looney).[147]

Creatine, unlike creatinine, is not an end-product of metabolism, nor is it a urinary constituent in the normal male adult. The concentration in the blood is usually about 4 mg. per 100 cc., more being present in the corpuscles than in the plasma. In severe nephritis, the content is somewhat increased though evidently this is not due to retention by the kidneys.

Glucose. The methods for determining blood sugar are based on the reducing action of glucose, under controlled conditions, using such reagents as an alkaline solution of copper tartrate. By Benedict's procedure,[148] which apparently excludes the non-sugar reducing substances, the normal blood-sugar level in the fasting state may be fixed at 70 to 90 mg. per 100 cc.[149]

The concentration of glucose in the corpuscle is less than in the plasma, but calculated on the basis of the water content of each, there is equal distribution between the cells and the plasma.

A man weighing 70 kg. has about 6 grams of glucose in circulation. When moderate amounts of carbohydrate are eaten, the sugar content of the blood in the portal vein increases appreciably, but in the systemic circulation little change is noted, owing to its rapid removal by the liver and other tissues. However, when more than moderate amounts of sugar are taken—50 to 100 grams—the sugar in the blood may increase to as much as 150 mg. per 100 cc., or even higher (see

[147] *J. Biol. Chem.*, **70**, 513 (1926).

[148] S. R. Benedict, *J. Biol. Chem* , **92**, 141 (1931). See also Folin and H. Malmros, *ibid.*, **83**, 121 (1929).

[149] Several methods in common use give somewhat different results. Actually, the results obtained by such methods are a measure, not only of the amount of glucose, but of other reducing substances as well. In order to differentiate between the so-called "true sugar" of the blood and other reducing constituents, especially non-sugars, advantage is taken of the fact that glucose is readily fermentable by yeast. Suitably prepared blood filtrates are analyzed before and after being subjected to the action of yeast, and in this way values are obtained for the total reducing substances (or apparent sugar) of the blood and for the non-sugar reducing substances. The difference between the two represents the concentration of glucose. According to Somogyi (*J. Biol. Chem.*, **75**, 33 [1927]; **78**, 117 [1928]), the amount of reducing non-sugars of the blood is very uniform, averaging 27 mg. per 100 cc. (expressed in terms of glucose). The concentration of "true sugar" in the blood, according to the data given by Somogyi, is frequently less than 90 mg. per 100 cc. In a group of 11 normal subjects, West (*J. Biol. Chem.*, **82**, 137 [1929]) obtained "blood sugar" values varying from 87 to 119 mg., of which 16 to 25 mg. represented non-sugar reducing substances. The "true sugar" varied between 68 and 94 mg. per 100 cc.

p. 318). The increase occurs during the first hour and is soon followed by a return of the blood sugar to normal levels.

Mention should also be made of the probable occurrence of hydrolyzable sugars in the blood. The analyses of Everett and Sheppard [150] indicate the presence of about 3 mg. of such carbohydrates per 100 cc. of plasma and about 10 mg. per 100 cc. of corpuscles.

The significance of data for blood sugar should therefore be clearly understood. Glucose predominates, but it is not the only reducing substance in the blood. In the present discussion the point may be emphasized that the glucose of the blood is on its way to the liver, muscles, and other tissues, either to be stored or oxidized. The blood going to the tissues (arterial blood) normally contains more sugar than the blood returning from the tissues (venous blood), as shown by the work of Foster [151] and others.

Blood Lipids. Blood plasma usually contains 0.5 to 0.8 per cent fat. The fatty acid content is from 0.2 to 0.45 per cent, being almost entirely in combination as phosphatide, neutral fat, and cholesterol esters. The total cholesterol normally varies between 150 and 200 mg. per 100 cc., but wider variations are not uncommon. It is about equally distributed between the cells and plasma; in the corpuscles the cholesterol is present in the free form, whereas in the plasma the greater part (50 to 80 per cent) is combined with fatty acids, as esters.

The following average data have been obtained by Boyd [152] in eight normal young women:

Total lipid, mg. per cent	589
Neutral fat, mg. per cent	154
Total fatty acid, mg. per cent	353
Phospholipid fatty acid, mg. per cent	130
Cholesterol ester, fatty acid, mg. per cent	77
Neutral fat fatty acid, mg per cent	146
Total cholesterol, mg. per cent	162
Combined cholesterol, mg. per cent	115
Free cholesterol, mg. per cent	47
Phospholipid, mg. per cent	196
Iodine number of total fatty acids	88.5
Iodine number of phospholipid fatty acids	124

The following physiological and pathological conditions are associated with an increased concentration of blood lipids (lipemia, or hyperlipemia) and usually by an increase in cholesterol (hypercholesterolemia): food ingestion, high fat diet, high meat diet, exercise, ether anesthesia, pregnancy, prolonged fasting, fever, diabetes, anemia, nephritis, nephrosis, hypothyroidism. Very high values for cholesterol, 500 mg. per 100 cc. of plasma, and above, are encountered not infre-

[150] *J. Biol. Chem.*, **80**, 255 (1928).

[151] *Ibid.*, **55**, 303 (1923); see also Friedenson, Rosenbaum, Thalheimer, and Peters, *ibid.*, **80**, 269 (1928).

[152] *J. Biol. Chem.*, **101**, 323 (1933).

quently in hypothyroidism and nephrosis. In hyperthyroidism, the cholesterol concentration tends to be low.

Lactic acid occurs normally in amounts of 10 to 20 mg. per 100 cc. of blood. In the resting individual the variation is from about 6 to 14 mg., with an average of 10 mg.[153] The concentration is increased with exercise and depends on its intensity. Very strenuous exercise may raise the concentration above 100 mg. The ingestion of carbohydrate, the administration of insulin, ether anesthesia, and pregnancy are associated with an increased content. High values have also been observed in heart failure, pneumonia, and tuberculosis.

Normal blood contains about 1 mg. per 100 cc. of the so-called *ketone bodies* (acetone, acetoacetic acid, and β-hydroxybutyric acid). The concentration is markedly increased in fasting individuals, particularly in children, in toxemias of pregnancy, persistent vomiting, and diabetes. In severe diabetes values of 300 mg. per 100 cc. have been recorded.

THE WATER BALANCE OF THE BODY

Water balance may be defined as the daily relation of the total amount of water entering the organism, through the intake of liquids and food, to the total output of water lost from the body by way of the kidneys, bowels, lungs, and skin. The water that results from the oxidation of the foodstuffs must also be included in the intake. Water is essential to life, and the supply, in most animals, must keep up with the demand. Voluntary abstinence from food for periods as long as two months has been endured by man, but deprivation of water for much shorter periods brings on serious effects. Rowntree cites the case of Viterbi, an Italian political prisoner who refrained from food and drink for eighteen days and died as a result. He is said to have suffered but little from hunger after the first day, but to have experienced terrible thirst until he died. Death from thirst, of individuals who are lost in the desert, is believed to result after 36 to 72 hours of water deprivation.[154]

It is estimated that from 7500 to 10,000 cc. of water per day are secreted into various parts of the alimentary tract as saliva, gastric juice, pancreatic juice, and intestinal juice. Nearly all this water, which is about two to three times the usual water intake, is reabsorbed. Water is lost to the body chiefly by way of the skin, respiratory tract, and kidneys. The kidneys are the most important, except under unusual conditions of heat and exercise, when the amount of water lost as sweat may exceed the amount in the urine.

Numerous attempts have been made to alter the composition of the

[153] A. V. Bock, D. B. Dill, and H. T. Edwards, *J. Clin. Investigation*, **11**, 775 (1932).

[154] The student is referred to W. B. Cannon, "The Physiological Basis of Thirst," *Proc. Roy. Soc. (London)*, *B*, **90**, 283 (1918).

blood by forced water administration. For example, in the experiment of Haldane and Priestley [155] 5500 cc. of water was taken in a period of four hours. Marked diuresis followed, so that at one time the rate of urine secretion was 2500 cc. per hour. And yet they observed no evidence of blood dilution. Similar results were obtained by Adolph.[156] These experiments show the remarkable control exercised by the kidneys in maintaining the normal water balance.

However, Greene and Rowntree,[157] and more recently, Calvin, Smith, and Mendel,[158] succeeded in producing blood dilution in the dog by forced administration of water. Similarly, the experiments of Margaria [159] on human subjects indicate that the blood may be definitely diluted as a result of drinking large quantities of water (p. 15).

Larson, Weir, and Rowntree[160] have described a form of water intoxication which may be produced in dogs, cats, rabbits, and guinea pigs by the administration of excessive amounts of water. The symptoms are nausea, vomiting, salivation, convulsions, stupor, and coma; death ensues if water administration is continued after the onset of the convulsions. On the other hand, the convulsions may be prevented by the injection of hypertonic saline when the first symptoms of the intoxication become apparent. The convulsions are believed to be cerebral in origin,[161] being related to disturbance of the water and electrolyte equilibria in the brain and the production of edema.

The factors of water retention and tissue hydration have also been associated with the convulsive seizures in epilepsy.

The water lost through evaporation constitutes 80 per cent, or more, of the so-called " insensible loss of weight." This is represented by the combined output of water vapor and carbon dioxide *minus* the weight of oxygen simultaneously absorbed. Under ordinary conditions, the insensible loss of weight of an adult during 24 hours may amount to about 1 kg.[162]

[155] *J. Physiol.*, **50**, 296 (1915–16).

[156] *Ibid.*, **55**, 114 (1921).

[157] *Am. J. Physiol.*, **80**, 209 (1927).

[158] *Ibid.*, **105**, 135 (1933); see also Chanutin, Smith, and Mendel, *ibid.*, **68**, 444 (1924).

[159] *J. Physiol.*, **70**, 417 (1930).

[160] Cited by Rowntree, *Physiol. Rev.*, **2**, 116 (1922).

[161] F. S. Smyth, W. C. Deamer, and N. M. Phatak, *J. Clin. Investigation*, **12**, 55 (1933), are inclined to relate the convulsive symptoms in water intoxication to the loss of chloride by way of the gastric secretion and to the resulting alkalosis.

[162] According to Benedict and Root, *Arch. Internal Med.*, **38**, 1 (1926), the insensible perspiration bears a quantitative relationship to the basal metabolic rate (p. 518) and may therefore be applied as an indirect method of determining the basal metabolism. This aspect of the subject, as well as the question of water balance, has received attention in various connections: M. W. Johnston and L. H. Newburgh, *J. Clin. Investigation*, **8**, 147, 161 (1929–30); F. H. Wiley and Newburgh, *ibid.*, **10**, 723 (1931); S. Z. Levine, J. R. Wilson, and M. Kelly, "The Insensible Perspiration in Infancy and Childhood," *Am. J. Diseases Children*, **33**, 204 (1927); **37**, 791 (1929); **39**, 917 (1930); I. McQuarrie, *et al.*, "Water Balance in Epilepsy," *ibid.*, **43**, 1519

There are many other aspects to the problem of water exchange, of which only a few can be referred to even briefly. The relation between water equilibrium and acid-base balance has been emphasized by a number of investigators.[163] Acidosis and dehydration are often associated phenomena, and alkalosis may be accompanied by sufficient water retention to show an increase in body weight.

Water retention due to lowered barometric pressure has been observed in rats and dogs by C. S. Smith.[164] Associated with this disturbance in water balance is marked restlessness, which has led to the interesting speculation of the possibility of a similar relationship existing in those animals which seem to have the ability to anticipate a change in weather.

The intravenous injection of strong hypertonic solutions of salt or glucose produces a marked lowering of the cerebrospinal fluid pressure, together with a diminution in the volume of the brain (Weed and McKibben).[165] These effects are brought about by the withdrawal of water from the brain, as well as from other tissues; they may also be obtained by the intra-intestinal administration of hypertonic solutions. Clinical application of these physiological principles has been made, particularly in brain surgery, where occasionally a modification of the pressure of the cerebrospinal fluid or a diminution of the brain bulk is desired.

A marked disturbance in water balance results from burns. The capillaries become abnormally permeable and a large proportion of fluid, rich in protein, is withdrawn from the circulation and localized in the tissues beneath the burned area. This is accompanied by a decrease in plasma protein, blood volume, and blood pressure. If the last is severe enough a condition of " shock " develops.[166]

The hormones of the adrenal cortex participate in the regulation of sodium and chloride metabolism and may have a direct effect on renal function, and therefore on the water output. In conditions of adrenal insufficiency (Addison's disease in man, extirpation of the adrenal glands in experimental animals), the normal relations of water and electrolytes may be maintained, at least for a time, through the administration of extracts of the adrenal cortex. However, when the

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(1932); *J. Nutrition*, **4**, 39 (1931). Methods of measuring the water exchange have been developed by L. H. Newburgh, M. W. Johnston, and M. Falcon-Lesses, *J. Clin. Investigation*, **8**, 161 (1929–30); P. H. Lavietes, *ibid.*, **14**, 57 (1935), and others.

[163] E. J. Stieglitz, *Arch. Internal Med.*, **41**, 10 (1928); W. McKim Marriott and A. F. Hartmann, *J. Am. Med. Assoc.*, **91**, 1675 (1928); L. Schoenthal, *Am. J. Diseases Children*, **37**, 244 (1929); Editorials in *J. Am. Med. Assoc.*, **90**, 1294, 1378; **91**, 2066 (1928); **92**, 148, 724, 898 (1929).

[164] *Am. J. Physiol.*, **87**, 200 (1928).

[165] *Am. J. Physiol.*, **48**, 512, 531 (1919).

[166] An experimental study of the mechanism of water exchange in the animal organism, accompanying burns, has been reported by Underhill, Kapsinow, and Fisk, *Am. J. Physiol.*, **95**, 302 *et seq.* (1930); see also Blalock *et al.*, *Arch. Surgery*, **22**, 598 *et seq.* (1931).

treatment is interrupted, the excretion of sodium and chloride is greatly augmented, this being accompanied by an increased urine volume. Indeed, the loss of water through the kidneys is often sufficient to produce concentration of the blood (hemoconcentration). Not only is there a loss of water from the plasma, but there occurs an even greater loss of interstitial body fluid. The sodium and chloride concentrations of the blood are diminished; the potassium is somewhat elevated, and the rise of blood urea is especially striking.

Regulation of water elimination by the pituitary and thyroid glands will be referred to in a later chapter.

Of importance to industry are the ill effects which are produced in individuals working at high temperatures and which recent work has associated with disturbance of the water-salt equilibrium. Of the various manifestations—heat prostration, heat pyrexia, and heat cramps—it is in the last condition that the relation seems to be most clearly defined. The concentrations of hemoglobin and plasma proteins are increased (evidence of hemoconcentration and probably of body-fluid depletion), while the concentrations of chloride and sodium are diminished (evidence of their depletion). The treatment is based purely on these physiological considerations (Talbott [167]) and involves the replacement of the salt and water lost as sweat during the working hours.[168]

Water and Temperature Regulation. Rowntree [169] states that water

[167] *J. Ind. Hyg. Toxicol.*, **19**, 258 (1937); *Medicine*, **14**, 323 (1935).

[168] The following vivid description by Haldane illustrates the effects of derangement of the salt and water balance:

"Perhaps the hottest place in England is about a mile under Salford, where the coal-miners work in boots and bathing-drawers, and empty the sweat from their boots at lunch—or snapping-time. One man sweated eighteen pounds in the course of a shift, and it is probable that even this figure has been exceeded. This sweat contained about an ounce of salt—twice what the average man consumes in all forms per day. The salt loss was instinctively made up above ground by means of bacon, kippers, salted beer, and the like. And as long as they did not drink more than a quart of water underground, no harm came to the miners. But a man who has sweated nearly two gallons is thirsty, and coal-dust dries the throat, so this amount was often exceeded, and the excess occasionally led to appalling attacks of cramp, often in the stomach, but sometimes in the limb or back. The victims had taken more water than was needed to adjust the salt concentration in their blood, and the diversion of blood from their kidneys to their muscles and skin was so great that they were unable to excrete the excess. The miners in question were offered a solution of salt in water which was about the composition of sweat, and would be somewhat unappetizing to the average man. They drank it by quarts and asked for more. And now that it has become their regular beverage underground there is no more cramp, and far less fatigue. It is almost certain that the cramp of stokers, and of iron and glass workers, which is known to be due to excessive water-drinking, could be prevented in the same way." J. B. S. Haldane, "Possible Worlds," Harper, New York and London, 1928, p 82. A fairly full account of "Some Effects of High Air Temperatures and Muscular Exertion upon Colliers" is given by K. N. Moss, *Proc. Roy. Soc., B*, **95**, 181 (1924).

[169] L. G. Rowntree, "The Water Balance of the Body," *Physiol. Rev.*, **2**, 116 (1922).

regulates heat distribution and dissipation because of its mobility and thermal properties. The high specific heat of water favors the storage of heat. The high caloric demands for the evaporation of water permit rapid elimination of heat when necessary. The high heat conductivity provides rapid equalization of heat within the tissues of the body, according to Barbour.[170] Rowntree points out that the latent heat of vaporization of water is of universal significance in relation to the dissipation of body heat, because of the fact that evaporation occurs at all temperatures.

Severe diuresis, owing to the depletion of water, produces a febrile condition in man and animals. In this way, Balcar, Sansum, and Woodyatt [171] produced a fever as high as 125.6° F. in dogs through the intravenous administration of concentrated solutions of glucose. An elevation in temperature also develops in infants deprived of food and water, but in new-born puppies Pucher [172] obtained an opposite effect, namely a rapid fall in temperature. In dogs, Greene and Rowntree [173] have observed a fall in temperature to result from the forced administration of water, even though the water given was at a temperature equal to or slightly above that of the body.[174]

THE LYMPH AND OTHER FLUIDS

The lymph is fundamentally a transudate, formed from the plasma by filtration through the capillary wall.[175] It therefore resembles the plasma in composition, except as this is modified by the impermeability of the capillary endothelium to protein. In accordance with Donnan's theory of membrane equilibria, differences in ionic concentrations (Na^+, H^+, Cl^-, HCO_3^-, etc.) are to be expected, but the non-electrolytes, such as sugar and urea, should be equally distributed between the two fluids, an equal distribution conforming with the idea that the lymph is a filtrate and not a secretion, as was supposed by many of the earlier physiologists.

[170] *Physiol. Rev.*, **1**, 295 (1921).

[171] *Arch. Internal Med.*, **24**, 116 (1919).

[172] *J. Biol. Chem.*, **76**, 319 (1928).

[173] *Am. J. Physiol.*, **80**, 230 (1928).

[174] Among the earlier reviews of the subject of water balance are those of Rowntree and of W. M. Marriott, *Physiol. Rev.*, **3**, 275 (1923). See also E. F. Adolph, *ibid.*, **13**, 336 (1933). The recent monograph of J. P. Peters, "Body Water," Thomas, Springfield, 1935, is especially recommended to the student. Consult also J. L. Gamble, *Bull. Johns Hopkins Hosp.*, **61**, 151, 174 (1937).

[175] The student is referred to the classical paper of E. H. Starling, *J. Physiol.*, **16**, 224 (1894), and to E. M. Landis, *Am. J. Physiol.*, **82**, 217 (1927).

A long series of important papers on lymph have appeared from C. K. Drinker's laboratory at Harvard. Many of these have been published in the *Am. J. Physiol.* since 1931. See also C. K. Drinker and M. E. Field, "Lymphatics, Lymph and Tissue Fluid," Baltimore, 1933.

The question of definition has been a source of confusion. Certain students of the subject hold the view that the term " lymph " should be restricted to the fluid within the lymph channels and should not be employed in designating the fluid which fills the intracellular spaces and in which the tissues are virtually bathed or "soaked." In line with the present conception that the lymphatics constitute a more or less closed system, it may be inferred that lymphatic lymph differs in chemical composition from tissue lymph. In truth, however, there is no evidence for this assumption. The peripheral lymphatics are relatively permeable and permit the free interchange of material, including protein, so that it is probably accurate to consider the two fluids similar in composition. Accordingly, in this brief discussion of the subject, extracellular tissue fluid, as well as the contents of the lymphatics, will be referred to as lymph.

Lymph collected from different regions of the body varies in composition. Thus, the fluid from the lower extremity contains 2 to 3 per cent protein; the lymph from the intestines 4 to 6 per cent, and that from the liver 6 to 8 per cent. These differences have been associated with variations in capillary permeability, rate of reabsorption, and other factors.

Considering the high concentrations of protein in lymph, it may be expected to exert an appreciable colloid osmotic pressure. Field and Drinker obtained values of 131 to 195 mm. H_2O for cervical lymph (dogs) and 138 to 344 mm. for thoracic duct lymph, the latter often containing a large amount of protein. In the same group of animals the values obtained for blood varied between 334 and 466 mm. H_2O. Drinker and associates hold the view that blood capillaries normally " leak " protein; this does not reënter the blood vessels unless delivered by the lymphatics.

The colloid osmotic pressure per gram of protein is higher for lymph than for blood. This accords with the relatively greater proportion of albumin in the former. It is the difference between the colloid osmotic pressure of the blood and lymph in a given area which determines the " effective " osmotic pressure of the blood and the return of fluid from the tissues.

A comparative study of the chemical composition of blood serum and thoracic lymph of the dog has been made by Arnold and Mendel,[176] whose results show that both normally and abnormally there is an interrelationship between the composition of the lymph and plasma. An interchange of material is continually taking place, and whenever any fluctuations in the concentrations occur, the diffusible constituents pass easily and rapidly between the blood, lymph, and the tissues. The normal relations are indicated in the following table:

[176] *J. Biol. Chem.*, **72**, 189 (1927).

TABLE XLIII

	Total Solids	Chlorides	Calcium	Phosphorus	Sugar	Non-protein Nitrogen	Protein Nitrogen
	G. per 100 cc.	Mg. per 100 cc.	Mg. per 100 cc.	Mg. per 100 cc.	Mg. per 100 cc.	Mg. per 100 cc.	G. per 100 cc.
Serum.....	8.3	392	10.4	4.3	123	27.2	0.9
Lymph.....	5.2	413	9 2	3 6	124	27.0	0 57

The relation of the composition of cervical lymph to that of the blood is indicated by the data in Table XLIV, obtained by Heim.[177]

TABLE XLIV

COMPARISON OF THE CONCENTRATIONS OF SOME OF THE CONSTITUENTS IN PERIPHERAL (CERVICAL) LYMPH AND BLOOD PLASMA OF THE DOG UNDER NORMAL CONDITIONS (NEMBUTAL ANESTHESIA)

	Protein (Kjeldahl)	N P. N.	Urea	Uric Acid	Creatinine	Sugar	Amino Acids	Chlorides as NaCl	Phosphorus		Calcium
									Total	Inorg.	
	Per cent	Mg per 100 cc	Mg per 100 cc.	Mg. per 100 cc	Mg per 100 cc.	Mg per 100 cc.	Mg per 100 cc	Mg per 100 cc	Mg per 100 cc.	Mg per 100 cc	Mg. per 100 cc.
Plasma: Average	6 18	32 6	21 7	Trace	1 37	123 0	4 90	678	22 0	5 6	11 70
Lymph: Average.... ..	3 32	34 8	23 5	Trace	1 40	132 2	4 84	711	11 8	5 9	9 84
Ratio: Lymph/plasma ..	0.54	1 07	1 07	...	1.03	1 08	0 99	1 05	0 54	1 05	0 84
Number of animals .	(16)	(10)	(7)	(3)	(7)	(16)	(1)	(7)	(6)	(3)	(11)

The lymph as it flows away from the lacteals is rich in fat and other absorbed material. As the fat is in a highly emulsified condition, the fluid has a milky appearance, and for this reason was called, by the earlier physiologists, "lacteal fluid," a name later replaced more or less generally by the term *chyle*. Except for a higher solid content, the chyle is very similar in composition to the lymph in other parts of the body.

Transudates and Exudates. The movement of fluid through the capillary wall is influenced by many factors. It has been shown, for

[177] *Am. J. Physiol.*, **103**, 553 (1933); see also A. M. Walker, *J. Biol. Chem.*, **101**, 269 (1933).

example, that above a venous pressure of 12 cm. H_2O, the rate of filtration is directly proportional to the increase in venous pressure, and when this reaches 15 to 20 cm., fluid tends to accumulate in the tissue spaces.[178] However, as the tissue pressure rises through the accumulation of fluid, it tends to diminish the filtration rate. The temperature of the part is also a factor, the rate of filtration through the capillary wall being greater at higher than at lower temperatures. Other factors have been considered elsewhere.

It must also be recognized that the permeability of the capillary endothelium is subject to change as a result of noxious influences, anoxemia, bacterial toxins, etc. Thus it is that the physical process of filtration associated with the normal exchange of fluid and other substances between the tissues and the blood may become modified particularly in the direction of increased accumulation of fluid in the tissues and body cavities (pleural, pericardial, peritoneal, etc.). When the process is non-inflammatory in origin, the product is called a transudate. Disturbance in circulation, with passive congestion, is a common cause. Inflammatory processes, on the other hand, give rise to exudates. As has been aptly stated by Wells,[179] it is often very difficult to decide whether a given fluid is an exudate or a transudate, there being no definite line of demarcation either etiologically or chemically. The differentiation is usually based on the following properties: (1) the specific gravity of transudates is below 1.015, that of exudates above 1.018; (2) exudates contain much more protein (3 per cent or above) than transudates; (3) transudates coagulate slowly, if at all, whereas exudates, because they contain much more fibrinogen, coagulate readily; (4) transudates are sterile, whereas exudates contain specific organisms; (5) exudates, in acute infections, contain pus cells (polymorphonuclear leukocytes); in chronic infections lymphocytes predominate; in transudates few cells are ordinarily present.

These differential points reveal a qualitative rather than a quantitative difference between exudates and transudates. We may consider first the condition in which the capillary endothelium is impermeable to protein. The resulting product is a protein-free filtrate, or ultrafiltrate, in short a transudate. The other extreme is the condition in which the membrane has become so altered as to permit the free passage of protein, so that the concentration of this constituent in the effusion is the same as in the blood. In general, exudates approach this condition. Transudates, on the other hand, represent a state in which the integrity of the capillary endothelium has not been reduced to the same extent and is therefore still capable of preventing to a greater or lesser degree the diffusion of protein, particularly the globulin and fibrinogen fractions.

The composition of a transudate while in equilibrium with the

[178] A. Krogh, E. M. Landis, and A. H. Turner, *J. Clin. Investigation*, **11**, 63 (1932); Landis and J. H. Gibbon, *ibid.*, **12**, 105 (1933).

[179] H. G. Wells, "Chemical Pathology," 5th edition, 1925, Chapter XVI.

blood is determined by known physicochemical laws. In studies of ascitic fluid [180] and edema fluid, the work of several investigators has established that the distribution of ions between the plasma and the transudate is governed by the Donnan equilibrium in the same manner as if the equilibration were established on the two sides of a collodion membrane. The protein content of ascitic fluid is variable. Loeb, Atchley, and Palmer [181] have recorded values of 0.8 to 0.9 per cent in three cases of hepatic cirrhosis, whereas, in a case of cardiac decompensation, as much as 4.5 per cent was found. As the amount of protein increases, the inequalities in ionic distribution between the plasma and transudate become less pronounced, but even in exudates, the Donnan effect is demonstrable.

According to the analyses of Greene and associates,[181] the total cation concentration is less in ascitic fluid than in serum, whereas the reverse holds for the anions. The accompanying data (Table XLV) are the averages obtained in a group of 10 patients. These values are expressed in milliequivalents per kilogram of water.

The water content of the ascitic fluid was, on an average, 96.74 per cent. The protein varied from 0.56 to 5.0 per cent; average 3.09 per cent.

TABLE XLV

COMPOSITION OF BLOOD SERUM AND TRANSUDATES (ASCITIC FLUID)

Na		K		Ca		Mg		Cl	
Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid
150 1	144	4 7	3 1	5	4	2 1	2 0	102 4	105 8

HCO_3		P		Total cations		Total anions	
Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid
29.5	28 7	2 2	2 1	161 9	153 4	136	138.8

Synovial Fluid. This is a viscid, transparent, alkaline fluid, contained in joint cavities, bursae, and tendon sheaths. Lubrication and

[180] Ascitis is an accumulation of serous fluid in the peritoneal cavity. Heart disease, cirrhosis of the liver, and kidney disease are the important general causes. Ascitis may also result from local causes, such as inflammation of the peritoneum, abdominal tumors, or obstruction to the portal circulation or of the inferior vena cava.

[181] Loeb, Atchley, and Palmer, *J. Gen. Physiol.*, **4**, 591 (1921–22); see also Hastings, Salvesen, Sendroy, and Van Slyke, *ibid.*, **8**, 701 (1927). Greene, Bollman, Keith, and Wakefield, *J. Biol. Chem.*, **91**, 203 (1931); Muntwyler, Way, and Pomerene, *ibid.*, **92**, 733 (1931).

the protection of the structures involved in movement are its principal functions. Motion is a physiological stimulus for increased production; inflammation, irritation, and trauma are pathological stimuli. Because of its accessibility, the fluid from the knee joint has been the most frequently studied.

Cajori and Pemberton [182] have compared the composition of blood plasma with that of synovial fluid in cases of arthritis with joint effusion and found almost identical values for non-protein nitrogen, urea nitrogen, and amino-acid nitrogen (Table XLVI). The concentration of non-electrolytes in synovial fluid may be changed by inducing corresponding changes in the blood. Less protein was present in the synovial fluid than in the plasma, and the albumin-globulin ratio was slightly higher in the synovial fluid than in the plasma. The sodium chloride content was somewhat higher in the synovial fluid. As pointed out by Fremont-Smith and Dailey,[183] in a similar study, these results may be explained by assuming that a simple membrane equilibrium exists between blood plasma and synovial fluid.

TABLE XLVI

CONCENTRATION OF DIFFUSIBLE CONSTITUENTS OF SYNOVIAL FLUID AND PLASMA IN CASES OF JOINT EFFUSION, IN MILLIGRAMS PER 100 CC. (AVERAGE OF 9 CASES)

Non-protein N		Urea N		Amino Acid N		Sodium chloride	
Plasma	Fluid	Plasma	Fluid	Plasma	Fluid	Plasma	Fluid
26 2	26 1	15 8	15 8	5 6	5 9	565	595

Cerebrospinal Fluid. Normal cerebrospinal fluid is a clear, colorless, limpid fluid with a specific gravity of 1.006 to 1.008. It has essentially the same freezing point as blood serum, indicating that the cerebrospinal fluid is in osmotic equilibrium with the blood. Cerebrospinal fluid and plasma have practically the same reaction (*p*H 7.35 to 7.40).

The protein content is very low, averaging about 25 mg. per 100 cc. with an albumin : globulin ratio of approximately 3 : 1. The concentration of calcium is usually 5 to 6 mg., or about 50 to 60 per cent of the serum concentration, and corresponds very closely to the amount of diffusible serum calcium. This suggests that cerebrospinal fluid is in the nature of a plasma dialysate, and indeed considerable data of the distribution of electrolytes (and non-electrolytes) have been submitted in support of this contention.[184]

[182] *J. Biol. Chem.*, **76**, 471 (1928); see also C. S. Keefer, W. K. Myers, and W. F. Holmes, *Arch. Internal Med.*, **54**, 872 (1934).

[183] *J. Biol. Chem.*, **70**, 779 (1926).

[184] F. Fremont-Smith, *Arch. Neurol. Psychiatry*, **17**, 317 (1927); Fremont-Smith, M. E. Dailey, *et al.*, *ibid.*, **25**, 1271, 1290 (1931); Dailey, *J. Biol. Chem.*, **93**, 5 (1931); E. Muntwyler, C. T. Way, and E. Pomerene, *ibid.*, **92**, 733 (1931).

Certain facts, however, oppose the theory that the spinal fluid is simply an ultrafiltrate. Although it is true that the glucose concentration runs more or less parallel in the plasma and the spinal fluid, the amount in the latter is normally considerably lower (about 65 mg. on the average). The point has been made that the tissues which are bathed by cerebrospinal fluid utilize the dextrose more rapidly than it is restored by the blood. However, Cockrill [185] has definitely shown that this inequality is not limited to glucose. Creatinine, urea, and uric acid are likewise unequally distributed between the water of the plasma and that of the cerebrospinal fluid. When plasma is dialyzed *in vitro* against cerebrospinal fluid, this unequal distribution of non-electrolytes is not obtained.

In experiments on frogs, Walker [186] has shown that the cerebrospinal fluid contains 30 per cent less of reducing substances than the plasma. The fluid also contains less phosphate. In depancreatized dogs with blood sugar values of 351 to 520 mg. per 100 cc., the cerebrospinal fluid contained from 220 to 292 mg. Walker has therefore concluded that cerebrospinal fluid is not formed by a simple process of filtration or dialysis, but that the choroidal epithelium possesses selective properties, not exhibited by the capillary epithelium or by the glomerular membrane.

A protein-free ultrafiltrate of plasma should fulfill the predictions based on Donnan's equilibrium. The concentration of chloride in the dialysate should be higher than in the plasma, while the concentration of base should be lower. This applies to cerebrospinal fluid only in a very general way. According to some of the data compiled by Flexner [187] (Table XLVII), the chloride concentration is higher, and the sodium is also higher. Less potassium is present in spinal fluid than in serum. It is also significant that, although the calcium content of spinal fluid is approximately equal to the diffusible calcium of serum, changing the concentration of the latter through the administration of calcium or of parathyroid hormone, or by removal of the parathyroids, does not seem to influence materially the calcium level of the cerebrospinal fluid.[188]

On the basis of such considerations, Peters [189] has expressed the opinion that the cerebrospinal fluid is not a protein-free ultrafiltrate of serum, but a much more highly differentiated fluid. The conception of cerebrospinal fluid as an ultrafiltrate is also rejected by Flexner in his review of the subject. A comparison of the concentrations of the various constituents of plasma and spinal fluid makes it apparent that work is performed in the production of the latter. Of the two possible sources

[185] *Arch. Neurol. Psychiat.*, **25**, 1297 (1931).
[186] *J. Biol. Chem.*, **101**, 269 (1933).
[187] *Physiol. Rev.*, **14**, 161 (1934).
[188] S. Morgulis and A. M. Perley, *J. Biol. Chem.*, **88**, 169 (1930); H. H. Merritt and W. Bauer, *ibid.*, **90**, 215, 233 (1931).
[189] J. P. Peters, "Body Water," p. 86.

of energy, the difference in hydrostatic pressure of capillary blood and spinal fluid does not account for more than a small fraction of the energy required. On the basis of certain thermodynamic considerations, Flexner has been driven to the conclusion that secretion is probably involved in the process of cerebrospinal-fluid production. By this is meant that the cells of the choroid plexus epithelium do not serve merely as a semipermeable membrane, but perform work in the formation of the fluid.

TABLE XLVII

COMPARISON OF CONCENTRATIONS OF CERTAIN CONSTITUENTS OF BLOOD SERUM AND CEREBROSPINAL FLUID*

Substance	Diffusible Moles (av.) per Liter Serum Water†	Moles (av.) per Liter Cerebrospinal Fluid
Amino acids	0 0046	0 0016
Creatinine	0 0002	0 00016 (?)
Uric acid	0 0003	0 00015 (?)
Urea	0 005	0 003
"Sugar"	0 0055	0 0048
Cl^-	0 106	0 12
$HPO_4^=$	0 001	0.0005
HCO_3^-	0 025	0 023
*p*H	7 35–7 40	7 35–7 40
Na	0 135	0 142
K	0 0043	0 0031
Mg	0 0006	0 0013
Ca	0 0015	0 0015
Lactic acid	0 0028	0 0022

* The values are based on the averages of data compiled and selected from the literature (after Flexner [187]).

† Calculated from assumed average serum water content of 0.14 cc. of water per liter of serum.

Intraocular Fluid. Krause [190] has systematically reviewed the chemistry of the aqueous and vitreous humors. In appearance and composition the aqueous resembles the cerebrospinal fluid very closely. It has a specific gravity of about 1.006 and a *p*H of approximately 7.4. The CO_2-tension is usually 65–70 volumes per cent. It contains about 20 mg. per cent of protein, and, as in cerebrospinal fluid, the concentration of calcium is equivalent to the diffusible calcium of the serum. The aqueous fills the anterior and posterior chambers of the eye and permeates the gel-like vitreous humor and the intracellular spaces of the various ocular coats. It is in osmotic equilibrium with the blood.

Duke-Elder [191] compared the composition of the blood serum and the intraocular fluids, obtaining the following results for sodium and chloride

[190] A. C. Krause, "The Biochemistry of the Eye," Baltimore, 1934.

[191] *Physiol. Rev.*, **14**, 483 (1934); see also Duke-Elder, "Recent Advances in Ophthalmology," Philadelphia, 1929, pp. 189–212.

(in millimoles per liter): aqueous, Na 121, Cl 123; vitreous, Na 119, Cl 117; serum, Na 145, Cl 103.[192] As a test of agreement with Donnan's theory of membrane equilibria, the values for aqueous and serum were substituted in the equation:

$$[Na^+]_{aqueous} \times [Cl^-]_{aqueous} = [Na^+]_{serum} \times [Cl^-]_{serum}$$

$$121 \times 123 = 145 \times 103$$

$$14{,}883 = 14{,}935$$

On the basis of these results and the close agreement of the glucose and urea concentrations of the blood serum and the aqueous which he obtained, Duke-Elder was led to believe that the aqueous humor is formed by a process of dialysis.

However, both the data and conclusions of Duke-Elder have been disputed. Walker [186] has reported differences in the composition of the aqueous and the serum of various animals (frog, fowl, rabbit, cat, and dog) and man. He found the non-electrolytes (urea, uric acid, sugar), as in cerebrospinal fluid, to be present in the aqueous in lower concentration than in the serum, or in ultrafiltrates derived from the serum. He therefore contended that the ciliary epithelium, like the choroidal epithelium, exhibits selective qualities not possessed by the capillary endothelium, or glomerular membrane, and that the intraocular fluids are not simple dialysates.

Walker's data for man are summarized in the following table:

TABLE XLVIII

	Reducing Substances, mg. per 100 cc.	Uric Acid, mg. per 100 cc.	Urea N, mg. per 100 cc.	Inorganic Phosphate-P, mg. per 100 cc.
Serum	99–113	2 2–5 1	17	2 8–4 1
Serum ultra filtrate	85–109		17	3 0–4 2
Aqueous humor	54–69	1 1–4 5	6	1 7–2 1

Peters [189] has also expressed the opinion that there is ample justification for considering the intraocular, as well as the cerebrospinal, fluid

[192] E. Tron, *Arch. Ophthalmol.*, **121**, 329 (1928), has submitted the following data for the distribution of ions between serum, aqueous and vitreous (in millimoles per kilo of water):

	Na	K	Ca	Mg	Cl	SO_4	HPO_4
Serum	156	7 95	5 6	1 33	112	1 87	3 23
Aqueous	147	4 87	3 1	0 875	123	0 75	1 77
Vitreous	147	4.87	3 4	0 8	125	1.1	0 65

as belonging to a different category from the intercellular or interstitial fluids proper, lymph, pleural and peritoneal fluids, and probably pericardial and synovial fluids. Those of the last group are all very similar in composition and resemble colloid-free ultrafiltrates of blood plasma, while cerebrospinal and intraocular fluids exhibit certain well-defined differences which make it very probable that they are not simple dialysates, but that secretion is an important factor in their production.

CHAPTER X

PHYSIOLOGICAL OXIDATIONS

The last two chapters have been concerned with the absorption of various types of molecules and the mechanism of their transport to the tissues of the body. In the next chapter we shall begin to consider the series of changes which these molecules undergo in the tissues. Here it is necessary to consider a most important aspect of these changes—the fact that they are attended by the liberation of energy, and that this energy is utilizable for work, mechanical, osmotic, or chemical, as for the synthesis of new cellular material.

The changes which the molecules undergo are both hydrolytic and oxidative. It is with the oxidative reactions that the most considerable and important liberations of energy are associated and with which we are concerned in this chapter. The few important changes associated with hydrolytic processes are left for subsequent consideration.

In order to integrate the discussion, a general outline of the above aspect (that is, the energy aspect) of the oxidative processes in the organism is first presented. This outline cannot be taken too rigidly. As will be seen, many points demand clarification by experimental work that is still to be done.

The outline follows; those terms which require and later receive explanation are italicized.

(1) An absorbed molecule after its transport to the tissues undergoes a series of step-by-step *oxidations* until it is finally converted into end-products.

(2) Some of these steps are what are known as *reversible processes*, and are therefore termed reversible *oxidation-reduction* systems.

(3) A reversible oxidation-reduction system possesses a certain *chemical potential*; in the laboratory, by the use of proper apparatus, such a chemical potential can be made apparent as an *electrical potential* or electromotive force.

(4) In the case of some *oxidant-reductant* pairs of substances found in the living cell, one member of the pair can be very readily converted to the other; in the case of other substances, however, such conversion demands the intermediation of various cell components (coenzymes, enzymes, etc.) which are often themselves readily reversible oxidation-reduction systems.

(5) When two reversible oxidation-reduction systems of different potentials interact, the one with the higher potential oxidizes the one

with the lower potential, and energy is liberated. This means that, in the system of lower potential, the equilibrium between the oxidant and the reductant shifts so that more of the substance is present as oxidant. Conversely, in the system of higher potential, as a result of the interaction, the equilibrium between the oxidant and the reductant shifts so that more of the substance exists as reductant.

(6) In the laboratory, this interaction of two oxidation-reduction systems of different potentials can be so arranged that the energy liberated appears in a *utilizable* form: electrical.

(7) In the cell, there also appears to be some contrivance, the nature of which is not yet known definitely, whereby the liberated energy appears in a utilizable form.

Reversible Systems. For a fuller discussion of this term, the student must consult a standard work on thermodynamics.[1] Here it may be stated that a reversible process is one in which the energy liberated is in a completely utilizable form, that no energy is dissipated in overcoming friction, electrical resistance, etc. For instance, a reversible chemical reaction,

$$A \rightleftharpoons A'$$

would be one in which, provided the appropriate apparatus was set up, the work necessary to change A' back to A was exactly equal to the work that was obtained when A changed to A'. Quite obviously such a process is an ideal one, but actual processes approach it to varying degrees, some quite closely.[2]

Oxidation. This term may be defined broadly in any of three ways:

(1) Addition of oxygen.
(2) Loss of hydrogen.
(3) Loss of electrons.

These definitions will assume more specific meanings in the course of the discussion. The processes noted are, of course, always accompanied by their converse. For example, if one substance in a system loses electrons, another component gains them. The *oxidant* is the substance which oxidizes; accordingly it is the component which loses oxygen or gains hydrogen or electrons. The *reductant* is the component which is oxidized; it gains oxygen or loses electrons or hydrogens.

The above definitions may be illustrated in the reaction:

$$Fe^{+++} + e \rightleftharpoons Fe^{++}$$

[1] G. N. Lewis and M. Randall, "Thermodynamics," McGraw-Hill Book Co., New York, 1923.

[2] Lewis and Randall state (p. 113): "An excellent example of an actual process which is very nearly reversible is furnished when the electromotive force of a galvanic cell is measured by means of a sensitive potentiometer. Here the driving force of the cell itself is so nicely balanced against an external electromotive force, that in favorable cases a current may be made to flow in one direction or the other by external changes of 0.00001 volt."

The ferric ion, Fe^{+++}, is the oxidant; it gains one electron. The ferrous ion is the reductant. When the reaction proceeds to the right, it is a reduction, since the concentration of oxidant decreases, and that of the reductant rises. Conversely, when the reaction proceeds to the left, it is an oxidation.

Oxidation-Reduction Potentials. We may begin our explanation of this term by an analogy to a mechanical system. When we say that a system has potential energy we conceive the situation that, when the appropriate means are supplied, work will be done. For instance, a stone on a shelf 8 ft. above the ground has potential energy; if allowed to fall off, the stone will do work in driving a wedge into the ground. If the stone is attached to a rope and pulley, it will do work by lifting another weight from the ground. A stone on a shelf 4 ft. above the ground has, of course, less potential energy than a similar one that is on the 8-foot shelf. The potential energy per unit of mass (in the case of the stone) is known as the potential.

Similarly when it is stated that an oxidation-reduction system possesses a given chemical potential energy, we conceive the situation that, when the appropriate means are supplied, the system will do work. Moreover, just as the stone changes its position from one where it has high potential energy to one where the potential energy is less, so we conceive the chemical system or some component of it to change from a condition of higher chemical potential energy to one of lower. The chemical potential is the potential energy per chemical equivalent.

The potential of an oxidation-reduction system may be made apparent in the laboratory by means of appropriate apparatus. Consider, for example, the reaction $Fe^{+++} + e \rightleftharpoons Fe^{++}$. A mixture of ferric and ferrous ions (as salts, of course) in a given ratio is placed in one vessel; another mixture of the two ions in a different ratio is placed in a second vessel. The two solutions are connected by means of a salt bridge. In each vessel is placed a metal strip of an indifferent metal—gold or platinum. Upon connecting these metal strips or electrodes by means of a wire, it is found that an electric current flows.

This flow of current may be explained as follows. The ferrous ions have a tendency to give off electrons. However, even when a few are given off, powerful electrostatic forces are set up which prevent further liberation of electrons and progress of the reaction. But when the electrodes, a joining wire, and the salt bridge are present, the electrons travel by way of the platinum and the wire from the solution where their liberation takes place with greater force to the solution where the force of liberation is less.

The passage of the electrons is manifested in the flow of current; this flow proceeds with an electromotive force which is the difference between the forces of liberation in the two solutions. The magnitude of this force can be determined experimentally by finding the magnitude of the counter electromotive force which must be sent into the circuit to stop

the current. This may be done by a potentiometric arrangement. In practice, the potential difference is measured not between two different mixtures of an oxidation-reduction system, but between a given mixture of the system and a standard hydrogen cell.

The following expression for the potential of an oxidant-reductant system may be derived on theoretical grounds; it has been verified experimentally in many cases.

$$E_h = E_0 + \frac{0.060}{n} \times \log \frac{\text{oxidant}}{\text{reductant}}$$

E_h is the potential in volts obtained at 30° when a mixture of oxidant and reductant is connected, in the way indicated, to a standard cell. E_0 is the potential obtained when the oxidant and reductant are present in equal amounts (a 50–50 per cent mixture) and is a characteristic constant. n is the number of electrons involved in the exchange. A reversible oxidation-reduction process can also take place in two successive steps. Each step has its characteristic oxidation-reduction potential. The theory of this type of reaction has been developed by several schools of investigators, notably by Michaelis and his coworkers.[3]

Effect of *p*H on the Potential of Oxidation-Reduction Systems. A component which acts as oxidant or reductant is very often in ionic form, the product of a compound which ionizes as an acid or base. Since the degree of ionization of acids or bases is affected by the hydrogen-ion concentration of the solution, the concentration of the ion which acts as oxidant or reductant is also affected. The *p*H therefore influences the ratio of the oxidant to reductant and, consequently, the potential dependent on this ratio.

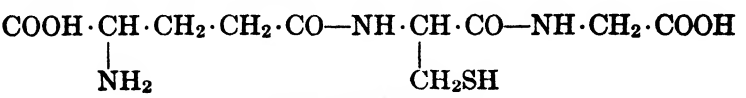
Naturally Occurring Oxidant-Reductant Systems. About 1920 several laboratories began the study of organic oxidation-reduction systems. That of W. M. Clark[4] has contributed especially to the determination of the potentials of many dye systems and to the elucidation of the rôle of various factors, such as *p*H, in connection with these potentials. Of most immediate concern are the oxidant-reductant systems which have been found to occur naturally. The values of the potentials of such systems furnish an idea as to the amount of energy

[3] *J. Biol. Chem.*, **91**, 335 (1931); **92**, 703 (1932); *J. Am. Chem. Soc.*, **53**, 2953 (1931). For greater detail concerning various aspects of this subject and the point of view here presented, the reader should consult the following: W. M. Clark, "The Potential Energies of Oxidation-Reduction Systems and Their Biochemical Significance," *Medicine*, **13**, 207 (1934); L. Michaelis, "Oxidation-Reduction Potentials," L. B. Lippincott Co., Philadelphia, 1930; B. Cohen, "Oxidations and Reductions," in B. Harrow and C. P. Sherwin's "Textbook of Biochemistry," W. B. Saunders, Philadelphia, 1935.

[4] "Studies on Oxidation-Reduction," 1-X, Hygienic Laboratory Bulletin 151, 1928; Studies XI–XVI, Supplements 61, 66, 69, 74, 92, U. S. Pub. Health Repts., 1927–1929.

that can be liberated in oxidation-reduction reactions and the extent to which such systems actually participate in physiological processes.

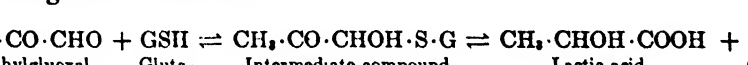
Sulfhydryl Systems. In 1921, Hopkins [5] isolated from yeast, muscle, and mammalian liver a substance which he named *glutathione*. This substance may exist in either oxidized or reduced form. The reduced form is a glutamic-cyst*eine*-glycine complex:



Glutamylcysteinylglycine
(Glutathione-reduced form)

It is the cysteine component which may be quite readily oxidized. The sulfhydryl grouping —SH— changes to a disulfide linkage —S—S— joining two molecules of reduced glutathione. Reduced glutathione, as well as its cysteine component, is readily oxidized by oxygen in alkaline solution. The determination of the potential of cysteine is attended with difficulty, and the mechanism of electron exchange appears to be different from that in the usual easily reversible dye system. Michaelis and Flexner [6] found the potential to be dependent on the concentration of cysteine, as well as on the usual factor of *p*H. At *p*H 7.4, 38° C., and 0.001 *N* concentration of cysteine, the potential was −0.272 volt. This marked negative potential agrees with the observed marked reduction power. Glutathione behaves in principle as does cysteine; some preliminary investigations by Michaelis indicate similar potentials.[7]

Though glutathione is widely distributed in the tissues, its rôle in physiological oxidations is not yet clearly known. It can reduce dehydroascorbic acid (oxidized ascorbic acid) to ascorbic acid (p. 304).[8] Lohmann [9] has shown that glutathione acts as a coenzyme for the conversion of methylglyoxal to lactic acid by the enzyme glyoxalase. Jowett and Quastel [10] have indicated that the above effect is due to the following series of reactions:



Methylglyoxal — Glutathione — Intermediate compound — Lactic acid — Glutathione

These reactions have been studied further.[11] An intermediate com-

[5] *Biochem. J.*, **15**, 286 (1921).

[6] *J. Biol. Chem.*, **79**, 689 (1928); see also L. Michaelis and E. S. G. Barron, *ibid.*, **81**, 29; **83**, 191 (1929).

[7] L. Michaelis, "Oxidation-Reduction Potentials," p. 142.

[8] H. Borsook, *et al.*, *J. Biol. Chem.*, **117**, 237 (1937); compare with F. G. Hopkins and E. J. Morgan, *Biochem. J.*, **30**, 1446 (1936).

[9] *Biochem. Z.*, **254**, 332 (1932).

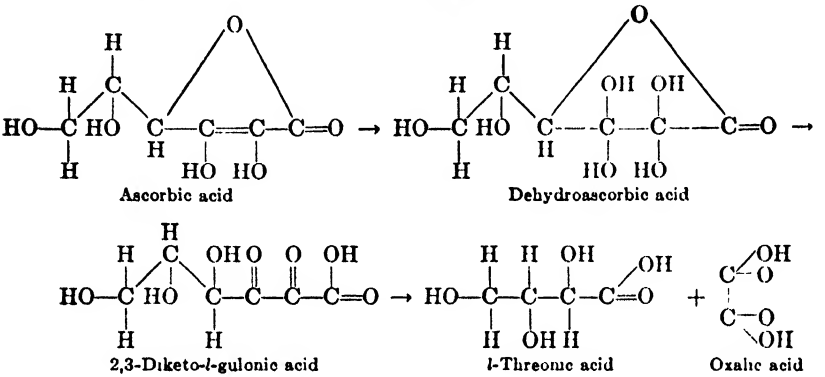
[10] *Biochem. J.*, **27**, 486 (1933).

[11] K. Nagaya, S. Yamazoye, and S. Nakamura, *J. Biochem. (Japan)*, **23**, 41 (1936).

pound of glutathione and methylglyoxal, as indicated above, has been isolated.[12]

In a previous chapter it was pointed out that the activity of some enzymes, e.g., urease, depended on the state of oxidation of the sulfhydryl groupings; the enzyme was inactive when these apparently were all converted to —S—S—.

Ascorbic Acid. The identity of this substance with vitamin C (p. 596) was established in 1933. Herbert, Hirst, and associates [13] have studied the oxidation products of ascorbic acid, and Borsook and his co-workers [8] have considered the following series:



They found the first stage, the oxidation of ascorbic acid to dehydroascorbic acid, to be reversible, and obtained a definite potential of 0.167 volt for a mixture of equal parts of oxidant and reductant at a *p*H of 4.0 and a temperature of 35.5° C. According to these workers, above *p*H 4.0, irreversible changes set in. The members of the first stage of oxidation, the ascorbic and dehydroascorbic acids, possess the same antiscorbutic potency, but the irreversibly oxidized products appear to possess none. Ball [14] also studied the potential of the ascorbic acid system and has found that more reliable values are obtained if these are determined in the presence of an easily reversible dye system.

Oxidized ascorbic acid (dehydroascorbic acid) is rapidly reduced by minced tissues—kidney, liver, intestinal mucosa. This action is as rapid in air as it is in vacuo and is due to the action of the glutathione present in these tissues.

Hemin Systems. Conant and his co-workers [15] have studied several of these systems. They found hemoglobin-methemoglobin to form a reversible system; the iron in the former compound was in the ferr*ous* state, and ferr*ic* in the latter compound. The determination of the

[12] S. Yamazoye, *ibid.*, **23**, 319 (1936).
[13] *J. Chem. Soc.*, Part 2, 1270 (1933).
[14] *J. Biol. Chem.*, **118**, 219 (1937).
[15] *Ibid.*, **57**, 401 (1923); **79**, 89 (1928); **98**, 57 (1932).

potential was difficult experimentally. At *p*H 7.0, E_0 (potential for an equal mixture of the reduced and oxidized forms) was found to be 0.152 volt. The potential for hemin at a *p*H of 9.15 and 22° was −0.230 volt, and considerably higher, 0.056 volt, for a pyridine-hemin compound.

As will be noted more fully later (p. 309), hemin compounds enter extensively into the stream of oxidative processes occurring in the cell. One of these most widely distributed compounds is *cytochrome*. Its presence has been established in aerobic bacteria, yeasts, higher plants, and animals. Because of the fact that the reduced form of cytochrome exhibits an absorption spectrum of four bands, whereas the oxidized form does not show any absorption spectrum, the oxidation and reduction of cytochrome may be observed directly in the living cells of small organisms.[16]

Cytochrome is a mixture of three independent hemochromogen-like compounds which have been designated *a*, *b*, *c*. Spectroscopic observations by several investigators indicate that the mixture and even the individual components may differ somewhat, depending on the tissue source.[17]

The most stable component of cytochrome, the *c*-component, has been studied in some detail. Theorell[18] has recently obtained very pure preparations. These showed an isoelectric point at about *p*H 9.7, a molecular weight of about 16,500, and an iron content of 0.34 per cent. Detailed spectroscopic examinations of these preparations were also made. One hundred kilograms of heart muscle yielded about 1 gram of a preparation of this degree of purity.

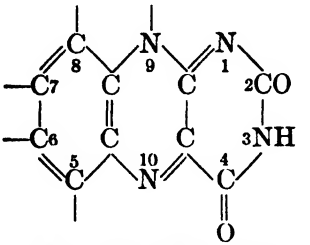
The rôle of cytochrome in oxidative processes is a prominent one and has been briefly discussed elsewhere (p. 250). Haas[19] has shown that the observed rate of reduction of cytochrome, as determined spectroscopically, corresponds to the entire respiration of baker's yeast. Coolidge[20] has studied the oxidation-reduction potential of cytochrome and has assigned a value for the potential of 0.260 volt for a 50 per cent reduced mixture at a *p*H of 7.0.

Natural Pigments. The study of natural pigments, other than the hemin compounds discussed above, which might engage in oxidation and reduction was begun by Cannan[21] with the investigation of hermidin from the plant *Mercurialis perennis* and of echinochrome from echinoderms. Since then a great many other pigments have been examined. A few of these may be mentioned: pyocyanine from *Bacillus pyocyane-*

[16] D Keilin, *Ergeb. Enzymforsch.*, **2**, 239 (1933).
[17] E. Elion, *Bull. soc. chim. biol.*, **18**, 165 (1936); H. Fink, *Z. physiol. Chem.*, **210**, 197 (1932); F. Urban, *J. Biol. Chem.*, **109**, xciii (1935).
[18] *Biochem. Z.*, **279**, 463 (1935); **285**, 207 (1936).
[19] *Naturwissenschaften*, **22**, 207 (1934)
[20] *J. Biol. Chem.*, **98**, 755 (1932).
[21] *Biochem. J.*, **20**, 927 (1926); **21**, 184 (1927).

ous, juglon from walnut shells, lawson from the henna plant, phthiocol from the tuberculosis bacillus.

The flavins are widely distributed in nature; they are found in yeast, milk, plants, and other tissues. All contain the flavin or isoalloxazine group, the formula of which is shown below.



Flavin or isoalloxazine nucleus

Lumilactoflavin is 6, 7, 9-trimethylflavin; *lactoflavin*, which is identical with *hepatoflavin* and vitamin B_2 (p. 591), and occurs in milk, plants, etc., is 6, 7-dimethyl-9-*d*-ribitylflavin; *cytoflav*, found in yeast, is lactoflavin-5′-phosphoric acid. All these substances have a rather negative potential, that is, E'_0 (potential at *p*H 7.0 and 50 per cent reduction mixture) is about −0.2 volt. The combination of lactoflavin-5′-phosphoric acid with a characteristic protein which constitutes the yellow ferment (p. 135) has, however, a distinctly higher potential, −0.06 volt at 38° and *p*H 7. The normal potential, at *p*H 7, of *toxoflavin*, found in cultures of *Bacterium bongkrek*[22] is −0.049 volt, and that of *hepatoflavin*, present in liver, is −0.24 volt. Kuhn and Boulanger[23] have synthesized many flavins and have studied the relation of chemical groupings in these compounds to their potentials.

Phosphopyridinenucleotides. The fact that certain relatively heat-stable and dialyzable substances are necessary for the action of enzymes involved in the oxidation processes of yeast fermentation or tissue metabolism has been appreciated for a considerable number of years. Such substances have been termed coenzymes. The nature of the one involved in yeast (cozymase) fermentation has been the subject of considerable investigation since 1929, when Euler and Myrbäck[24] obtained evidence indicating that it contained phosphorus, adenine, and a pentose. Recently, Euler and his co-workers[25] made a most distinct advance in identifying the cozymase as a diphosphopyridine nucleotide containing a nicotinic acid amide nucleus. The probable structure has

[22] K. G. Stern, *Biochem. J.*, **29**, 500 (1935); F. G. Stare, *J. Biol. Chem.*, **112**, 223 (1935); R. Kuhn and G. Moruzzi, *Ber.*, **67**, 1220 (1934).

For further details concerning this organism and its pigment, see Van Veen and Mertens, *Rec. trav. chim.*, **53**, 257, 398 (1934), and the article by K. G. Stern.

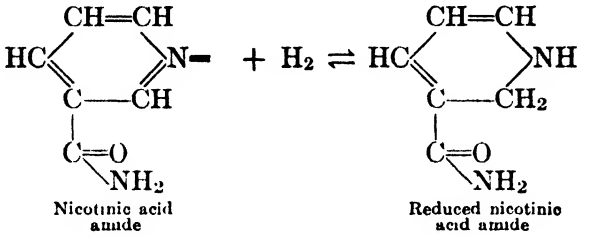
[23] R. Kuhn and P. Boulanger, *Ber.*, **69**, 1557 (1936).

[24] *Z. physiol. Chem.*, **184**, 163 (1929).

[25] *Ibid.*, **237**, 1 (1935); **238**, 233, **240**, 113, **241**, 239, **242**, 215 (1936).

been considered in a previous chapter (p. 154). Meanwhile, Warburg and his co-workers [26] had been studying the nature of the system present in red-blood cells and elsewhere which was effective in oxidizing hexosephosphate. They found that a coenzyme was also necessary in this process and finally identified this as triphosphopyridinenucleotide.

The important and striking feature in these findings is that the two coenzymes contain a common chemical grouping which constitutes a *readily reversible oxidation-reduction system.* This may be pictured as follows:



Nicotinic acid amide — Reduced nicotinic acid amide

The reduced form of cozymase, now also designated as coenzyme I, or *DPN* (*d*iphosphop*y*ridine*n*ucleotide), shows a white fluorescence, and a characteristic absorption band at 340 $m\mu$. It is possible to follow the progress of dehydrogenation spectroscopically. Warburg's coenzyme has been designated coenzyme II, or *TPN* (*t*riphosphop*y*ridine*n*ucleotide). The possibility exists that coenzymes necessary in other types of oxidative reactions possess similar structures.

The Interaction of Metabolites and Molecular Oxygen. We have just seen that there are in the cells of various types of organisms readily reversible oxidation-reduction systems: nitrogenous hemin compounds, the yellow enzyme, other flavins such as hepatoflavin, hermidin, echinochrome, pyocyanine, glutathione, and ascorbic acid. The question now arises: what relation do these readily reversible systems bear to the oxidative changes that come to our attention in the course of metabolism —the changes, for example, of hexosephosphoric (p. 323) to phosphohexonic acid (product formed by the oxidation of the aldehyde group of hexose phosphate to the carboxyl), of lactate to pyruvate, of succinate to fumarate?

Let us consider this last example. A solution of succinate, freely exposed to the air, does not interact with oxygen. If, however, an extract of minced muscle which has previously been washed with water is added to the solution of succinate, oxygen is used up, succinate disappears, and fumarate is formed.

Such a reaction may, of course, be designated in a general way as an enzymic one. But attempts to determine to some degree the nature of the "enzyme" concerned in the above interaction of succinate, or other metabolites with molecular oxygen, have revealed that the "enzyme"

[26] *Biochem. Z.*, **282**, 157 (1935).

consists of more than one component and that some or all of these components can be shown to be or to resemble readily reversible oxidation-reduction systems of the kind that have been discussed.

There are, however, other enzymes mediating the interaction of metabolites with molecular oxygen which so far have not been shown to consist of more than one component. This, of course, does not exclude the possibility that such enzymes may be plural in composition, the components simply being more firmly combined than in the type of enzymes first mentioned.

Examples of enzymes which mediate the interaction between metabolites and molecular oxygen and which so far have not been shown to consist of more than one component follow:

A. Uricase.[27] Minced and ground kidney or liver tissue is extracted with acetone; the insoluble residue is washed with acetone. The powder thus obtained mediates the interaction between uric acid and molecular oxygen; allantoin and carbon dioxide are formed (p. 436).

B. Amino Acid Oxidase.[28] This may be prepared in the same way as the uricase above. The following reaction occurs in its presence:

$$R \cdot CH(NH_2) \cdot COOH + \tfrac{1}{2}O_2 \rightarrow R \cdot CO \cdot COOH + NH_3$$

C. Tyrosinase.[29] This enzyme may be obtained from potatoes, mushrooms, wheat bran. Its best-known action is the oxidation of tyrosine to the pigment melanin. However, the presence of this enzyme also mediates the interaction of monohydric phenols with molecular oxygen to form orthodihydroxy compounds, and the further interaction of the latter to form orthoquinones.

We shall now give a few examples of metabolites which interact with molecular oxygen in the presence of tissue preparations that constitute enzyme systems of more than one component.

A. The Interaction of Lactic Acid with Molecular Oxygen to Form Pyruvic Acid. The *α-Hydroxyoxidase System of Gonococci* (Barron and Hastings [30]). The enzyme preparation is a cytolyzed suspension of washed gonococci. The action of two components has been demonstrated. The first is not destroyed by heating for 2 hours at 50°; its activity is not affected by HCN or H_2S. Its purpose, according to the authors, is to activate the lactic acid, producing a rearrangement of the electronic structure of the lactic acid so that it becomes electromotively active. The second component is inactivated by HCN or H_2S, and is destroyed by heating at 50° for 2 hours. When the second component is thus destroyed or inactivated, no interaction with oxygen occurs. How-

[27] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London), B*, **119**, 114 (1936).

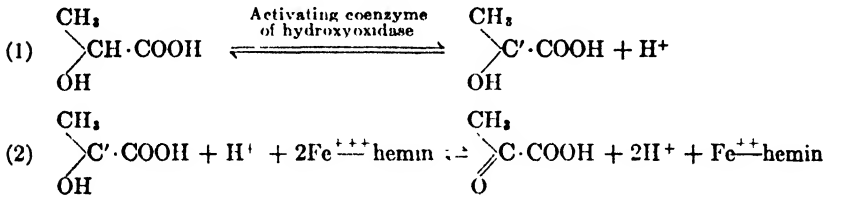
[28] H. A. Krebs, *Z. physiol. Chem.*, **217**, 351 (1933); *Biochem. J.*, **29**, 1620 (1934).

[29] C. E. M. Pugh and H. S. Raper, *Biochem. J.*, **21**, 1370 (1927); M. Graubard and J. M. Nelson, *J. Biol. Chem.*, **111**, 756 (1936).

[30] *J. Biol. Chem.*, **100**, 155 (1933).

ever, the second component, so destroyed or inactivated, may be replaced by a reversibly oxidizable dye, to some extent by hemin, and much better by nicotine-hemin.

The second component is therefore considered to be of the nature of a ferric nitrogen hemin compound, of relatively high reduction-oxidation potential. After activation of the lactic acid, this second component and the activated lactic acid exchange electrons. The entire scheme of oxidation may be represented as follows:



The last equation represents the interaction of the ferrous hemin compound with the molecular oxygen. It should be noted that the activating factor in this system (the first component) has not yet been subjected to any chemical analysis.

B. Interaction of Succinic Acid with Molecular Oxygen to Form Fumaric Acid. Heart Muscle Extract as Enzyme System (Stotz and Hastings [32]). The formulation of this reaction is similar to the one above. The enzyme preparation also consists of two components. The first activates the succinic acid, and is termed the dehydrogenase factor by the authors. It is completely inhibited by selenite and is more easily destroyed by heat than the activating factor in the lactate system discussed above. The second component is designated the oxidase factor; it is completely inhibited by cyanide and is replaceable by reversible dyes. The extent of the replacement depends on the oxidation-reduction potentials of these dyes.

C. The Interaction of Hexosemonophosphoric Acid with Molecular Oxygen to Form Phosphohexonic Acid. Warburg and Christian's Enzyme

[31] The last two stages in the cycle of reactions may be pictured as:

$$2H + 2\,O \rightarrow H_2O_2$$

$$H_2O_2 + \text{catalase} \rightarrow H_2O + \tfrac{1}{2}\,O_2$$

In the paper by Barron and Hastings the chain of reactions is represented as follows:

(1) $2CH_3CHOHCOO^- + 4Fe^{+++} \rightleftharpoons 2CH_3COCOO^- + 4Fe^{++} + 4H^+$
(2) $4Fe^{++} + 2O_2 \rightleftharpoons 4Fe^{+++} + 4O^-$
(3) $4H^+ + 4O^- \rightarrow 2H_2O_2$
(4) $2H_2O_2 + \text{catalase} \rightarrow 2H_2O + O_2$

[32] *J. Biol. Chem.*, **118**, 479 (1937).

System (Warburg, Christian and Griese [33]). This enzyme system consists of three components. The first is a coenzyme, obtainable from red blood cells. It has been shown to be a triphosphopyridinenucleotide, capable, as has already been stated, of being reversibly oxidized and reduced. The second component has been designated as *Zwischenferment* or "intermediate enzyme" and has been purified to a great extent by Negelein and Gerischer.[34] It is a protein, obtainable from yeast, and has apparently no enzymic property of its own; its presence, however, is necessary for the action of the coenzyme. The third component is the "yellow enzyme, or ferment." As has already been stated (pp. 135, 155), this is a flavoprotein and contains an isoalloxazine nucleus which is also capable of acting as a reversible oxidation-reduction system.

The reaction due to the above system may be pictured as follows:

(1) Coenzyme + hexosemonophosphoric acid $\xleftarrow{\text{Zwischenferment}}$ reduced coenzyme + phosphohexonic acid

(2) Reduced coenzyme + yellow enzyme $\rightleftharpoons$ coenzyme + reduced yellow enzyme

(3) Reduced yellow enzyme + molecular oxygen $\rightleftharpoons H_2O_2$ + yellow enzyme

It is of interest to consider reaction (1) in more detail. If large and equimolecular amounts of coenzyme and hexosemonophosphoric acid are present together with Zwischenferment, reaction (1) proceeds to a very noticeable degree. If, however, only a very small amount of coenzyme, relative to the amount of hexosemonophosphoric acid, is present—e. g., in the ratio of 0.00005 mole of coenzyme per mole of substrate—then only 0.00005 mole of reduced coenzyme is formed, and, to all appearances, no noticeable reaction occurs. If, at this stage, the yellow enzyme is added, reactions (2) and (3) are initiated; the 0.00005 mole of reduced coenzyme is reconverted into the oxidized form and is able to react again according to reaction (1). In fact, the coenzyme is able to pass through this cycle 20,000 times producing the effect of 1 mole of coenzyme.

This consideration is important when we dwell on the idea of the "activation factor" which is a prominent feature in the elucidation of the mechanism of several enzyme systems. The above experiment, for instance, might be interpreted as an "activation" of the substrate by a small amount of coenzyme, were we not in a position to have the coenzyme in a relatively pure form, to study the reaction between it and the substrate in stoichiometric ratios, and to realize, as Warburg and his co-workers have pointed out, that the "activation" is merely a rapid and repeated turnover of a small amount of a reversibly oxidizable phos-

[33] *Biochem. Z.*, **282**, 157 (1935).
[34] *Ibid.*, **284**, 289 (1936).

phopyridinenucleotide. The possibility exists that a similar mechanism operates in other kinds of reported activations.

D. The Interaction of Lactate with Molecular Oxygen to Form Pyruvic Acid. Enzyme System of Green and Brosteaux.[35] This system also consists of three components, and a close analogy to the Warburg, Christian, and Griese system has been drawn. The first component is an enzyme from heart muscle; the second component, a coenzyme, is obtained from baker's yeast, or from muscle. The third component is a reversible dye like methylene blue or pyocyanine. Yellow enzyme or adrenaline may also be used as the third component, but cytochrome, glutathione, or ascorbic acid cannot substitute for it. The reaction may be pictured as follows:

(1) Lactate + coenzyme $\xrightleftharpoons{\text{Enzyme of heart muscle}}$ pyruvate + reduced coenzyme

(2) Reduced coenzyme + reversible dye $\rightleftharpoons$ coenzyme + reduced dye

(3) Reduced dye + molecular oxygen $\rightleftharpoons$ oxidized dye

The coenzyme used in the above reaction closely resembles the coenzyme of the Warburg, Christian, and Griese system.

The above examples of the interaction of metabolites with molecular oxygen through the intermediation of systems of cell components are representative of other such interactions which at present are being elucidated. Though such systems have been classified in various ways (see, for example, Green and Brosteaux [35]), the fundamental feature of all these systems appears to consist in a chain of components, some of which are not yet precisely known, acting together.

Catalase and Peroxidase. Catalase decomposes hydrogen peroxide into oxygen and water, and also acts on other peroxides. The advances regarding its chemical nature, reported by Stern [36], have been discussed in a previous chapter (p. 134). Its occurrence in plant and animal tissues is widespread. It may be noted that, according to some of the representations for the interaction of the various metabolites with molecular oxygen, hydrogen peroxide is formed. The function of catalase in physiological oxidations is thereby indicated.

Keilin and Hartree [37] have also recently noted that hydrogen peroxide is found in oxidation reactions catalyzed by xanthine oxidase, uricase, and amino-acid oxidase. They studied the oxidation of alcohol which was promoted by the above systems and made the formulation that such a coupled oxidation required: (1) a peroxide in a "nascent" state and presumably formed in the above oxidation reactions; (2) some factor, most likely in the catalase preparation, which activates the peroxide so that it can oxidize the alcohol.

[35] *Biochem. J.*, **30**, 1489 (1936).
[36] *Ibid.*, **30**, 629 (1936).
[37] *Proc. Roy. Soc. (London), B*, **119**, 141 (1936).

Peroxidase is widely distributed in plant and animal tissues. It is especially abundant in the roots and seedling sprouts of the higher plants. In the presence of hydrogen peroxide, or other peroxides, and this enzyme, oxidation of various organic substances occurs. The best known are the oxidation of guaiaconic acid, tyrosine, and pyrogallol. The familiar bluing of gum guaiac when mixed with potato scrapings is explained by the interaction of the guaiaconic acid present in the gum, and a peroxide and peroxidase present in the potato scrapings. The details of the mechanism of peroxidase action, however, have not yet been elucidated.

Cell Oxidations as a Source of Utilizable Energy. It is now necessary to return to the main problem of this chapter. How do the oxidations in the cell provide a source of energy which is utilizable for the various types of work the organism performs?

We have seen that the study of the interactions of cell metabolites with molecular oxygen indicates that such interaction, in the instances given, is accomplished by a system of cell components which constitutes a *bridge* of reversible oxidation-reduction reactions, each a source of energy, and each reaction corresponding, as it were, to a span of the bridge.

Electromotive measurements of the metabolites which interact with molecular oxygen are very instructive in this respect. A mixture of lactate and pyruvate, when connected to a standard hydrogen cell, shows no potential indicative of electron transfer.[38] When α-hydroxyoxidase is added to the lactate-pyruvate mixture, an erratic potential is obtained, showing some tendency for electrons to move along the platinum electrode-wire connection. When, in addition, a reversible dye is added, a stable potential is obtained within 1 to 2 hours. In short, it may be said that the addition of the enzyme system and the dye supplies several additional spans to form a bridge over which the electrons travel from one cell to the other. A similar, though not as complete, picture can be drawn of the interaction between metabolites and molecular oxygen in the test-tube. For example, triphosphopyridinenucleotide accepts hydrogen (or electrons) from hexosemonophosphoric acid and then transfers it to the yellow enzyme; the yellow enzyme in turn gives up the hydrogen which reacts with the molecular oxygen.

In the laboratory, we have seen, the interaction of two reversible oxidation-reduction systems of different potentials may be so arranged that the energy is liberated in a form (electrical) utilizable for work. The interaction may be conceived of as a stream of electrons which travel over the platinum electrode-wire from the system of lower potential to the one of higher. The question arises as to what, in the living cell, corresponds to this platinum-wire arrangement in the laboratory.

It has been noted that the system of cell components which mediate

[38] E. S. G. Barron and A. B. Hastings, *J. Biol. Chem.*, **107**, 567 (1934).

the interaction of a metabolite with molecular oxygen can be conceived of as a bridge over which hydrogen passes from the reductant to the oxidant. Now, if one or more of these mediators can be thought of as extending or moving from one locus in the cell to another, we have a way of explaining how a mediator may carry energy from a region where it is liberated to one where it is absorbed in the course of another reaction. This concept, suggested by Quastel,[39] has been extended by Schott and Borsook [40] and by Dewan and Green.[41]

[39] *Biochem. J.*, **20**, 166 (1926).
[40] *Science*, **77**, 589 (1933).
[41] *Biochem. J.*, **31**, 1069 (1937).

CHAPTER XI

INTERMEDIARY METABOLISM OF CARBOHYDRATES

In tracing the fate of carbohydrates in metabolism, we are primarily concerned with the chemical changes which glucose undergoes after absorption. The carbohydrates of the diet are not the only source of glucose, for certain of the amino acids and glycerol are convertible into sugar and glycogen. In 100 grams of protein there is a sufficient amount of the so-called sugar-forming amino acids to yield about 58 grams of glucose.[1] As we shall see in a later chapter, the stages in the intermediary metabolism of these amino acids are in part similar to those encountered in carbohydrate metabolism. The actual formation of glucose from protein can be demonstrated when little or no carbohydrate is fed, during starvation, in pancreatic diabetes, and in phlorhizin diabetes. The last is a severe form of renal glycosuria produced by injecting phlorhizin (p. 60) into animals. Dogs made "diabetic" in this way may excrete large amounts of sugar even after the glycogen of the liver has been exhausted. In these animals the proteins of the food and of the tissues are partly converted to glucose, and, if the disturbance is severe enough, for each gram of nitrogen excreted in the urine, 3.65 grams of glucose are simultaneously eliminated. Each gram of nitrogen in the urine represents the metabolism of about 6.25 grams of protein.

A certain amount of glucose is derived from the glycerol part of the fat molecule. Chambers and Deuel [2] have shown a practically complete conversion of glycerol to glucose in phlorhizinized dogs. Ordinarily, glycerol is oxidized to carbon dioxide and water. The majority oppose the view that fatty acids are convertible into carbohydrate; however, the possibility that this may occur under certain conditions cannot be dismissed. Because of conflicting experimental data and lack of agreement in their interpretation the question has become exceedingly controversial. It will be considered in somewhat greater detail in another connection (p. 366).

Glycogen Synthesis. The sugar entering the portal circulation is largely deposited in the liver as glycogen. Normally the liver also

[1] The amount of sugar obtainable from protein varies somewhat with different proteins, depending on the relative proportion of sugar-producing amino acids. For details see D. Rapport, "The Interconversion of the Major Foodstuffs," *Physiol. Rev.*, **10**, 349–472, and especially p. 392 (1930).

[2] *J. Biol. Chem.*, **66**, 21 (1925).

removes glucose from the systemic blood when its concentration exceeds a certain level (about 70 to 90 mg. per cent). The process of glycogen formation, or *glycogenesis*, in the liver, in addition to conserving food material for subsequent utilization, thus also serves in the regulation of the sugar concentration in the blood.

Fructose is converted into glucose and glycogen in the liver, the rate of glycogen formation being even greater than that for glucose, while galactose is stored more slowly, or less completely, so that when a large amount of this sugar is fed, a considerable proportion is excreted in the urine.[3] In man, the pentose sugars are not utilized, and when fed are excreted unchanged. It has also been shown (in the rat) that xylose does not form glycogen.[4] After its oral administration, a certain amount is retained in the liver, blood, and kidney, but it is probable that the pentose is only temporarily stored and is eventually excreted unchanged

Herbivorous animals obtain a certain amount of their energy from pentosans. The polysaccharides are acted upon by bacteria in the alimentary tract. By this action a variety of substances, such as organic acids, are produced which the animal is able to utilize. The synthesis of lactose from the fermentation-digestion products of the pentosans has also been suggested. However, in man, the pentosans are not utilized at all, and if pentose sugars are fed, they are excreted unchanged in the urine.

The following amino acids are considered to be potential sources of glycogen in the animal organism: glycine, alanine, serine, cystine, aspartic acid, glutamic acid, hydroxyglutamic acid, arginine, and proline. In experiments with white rats that had been previously fasted for 24 hours, Wilson and Lewis[5] demonstrated the deposition of glycogen as a result of the oral administration of *d*- and *dl*-alanine. Contrary results were obtained with two other sugar-forming amino acids, glycine and *d*-glutamic acid, which yielded only small amounts of glycogen, as determined by comparing the composition of the livers of the fed animals and those of suitable controls.

In the fasting animal about 10 per cent of the fat, representing the glycerol, is a potential source of glucose and glycogen. Catron and Lewis[6] fed rats that had been fasted for 24 hours 1 gram of glycerol. This was followed during the next 2–3 hours of observation by an increase in the glycogen content of the liver, comparable to that obtained on feeding an equivalent amount of glucose.

As will be described later, lactic acid is another source of liver glycogen. The conversion in the liver of *d*-lactic acid (as the sodium

[3] The intermediary metabolism of fructose and galactose has been reviewed by H. J. Deuel, *Physiol. Rev.*, **16**, 173 (1936).

[4] M. M. Miller and H. B. Lewis, *J. Biol. Chem.*, **98**, 133, 141 (1932).

[5] *J. Biol. Chem.*, **84**, 511 (1929); compare with H. J. Deuel, *Ann. Rev. Biochem.*, **6**, 229 (1937).

[6] *J. Biol. Chem.*, **84**, 553 (1929).

salt) into glycogen has been observed by Cori and Cori[7] in experiments conducted on the white rat. In contrast, *l*-lactic acid, though absorbed at the same rate, formed practically no liver glycogen and about 30 per cent was excreted in the urine. In the opinion of these investigators, *l*-lactic acid is utilized, but only about one-fourth as readily as the dextro isomer.

Glycogenesis in Muscle. Muscle glycogen is formed principally from the glucose of the blood. It is stated that the glucose concentration is usually greater in arterial than in venous blood, owing to the withdrawal by the tissues of some of the sugar. This is especially evident after a carbohydrate-rich meal.

Although the ingestion of fructose leads to an increase in muscle glycogen, this is not due to its conversion in the muscle tissue itself. Inasmuch as the liver is capable of transforming fructose into glucose, it is conceivable that muscle glycogen owes its origin to the glucose thus formed. However, even when the liver is completely extirpated, injected fructose leads to the deposition of glycogen in muscle. This would be convincing proof of a fructose → glycogen synthesis, but for certain facts reported by Bollman and Mann.[8]

These investigators found that, in hepatectomized dogs, the injection of fructose was followed by a rise in the glucose content of the blood, but if, of all the remaining viscera, the intestines alone were also removed, this rise did not occur. Nor was any muscle glycogen formed under these conditions. Bollman and Mann have shown further that in animals which had intact livers, but from which the stomach and intestines had been removed, the process of conversion of fructose to glucose is apparently possible.

In short, fructose is not utilized directly by muscle in the synthesis of glycogen. It is first converted into glucose, either in the intestines or liver, but not, to any appreciable extent, in other tissues of the body.

Synthesis and storage of glycogen in the liver are believed to be controlled by insulin, the pancreatic hormone (p. 465), but the underlying mechanism is obscure. It is not even known whether the effect of insulin is primarily to promote glycogen synthesis, or to inhibit the reverse process of *glycogenolysis*. The great reduction of the glycogen reserve of the liver in diabetes has been associated with the diminished amount of insulin secreted by the pancreas. Moreover, the administration of insulin to diabetic animals has been found to increase the amount of glycogen in the liver. There is, however, the possibility that insulin, as well as other factors external to the liver (diabetogenic hormone of the pituitary, adrenaline, etc.), are of secondary, rather than of primary importance. The work of Soskin[9] and associates indicates that the

[7] *Ibid.*, **81**, 389 (1929); compare with Abramson, Eggleton, and Eggleton, *ibid.*, **75**, 763 (1927).

[8] *Am. J. Physiol.*, **96**, 683 (1931).

[9] *Am. J. Physiol.*, **109**, 155; **110**, 4 (1934); **114**, 648 (1935–36); **119**, 407 (1937).

mechanism regulating retention and output of carbohydrate in the liver may reside in the liver itself.

Less is known of the rôle of insulin in the glycogen metabolism of muscle. According to certain authorities there is no convincing evidence that it has anything to do either with the synthesis or storage of muscle glycogen. Nor has it been established that the hydrolysis of muscle glycogen is increased in the absence of insulin. It may be shown, however, that the content of glycogen in muscle is increased in the diabetic animal (and also in the normal) by the administration of insulin especially when glucose is also provided.

Glycogenolysis. To meet the requirements of the tissues when no carbohydrate is being absorbed, the glycogen of the liver is converted into glucose, a process termed *glycogenolysis*. The importance of the liver as a constant source of supply of blood sugar is shown by the fact that, when this organ is extirpated, the blood-sugar concentration diminishes rapidly and the animal dies. No other organ or tissue of the body seems capable of supplying carbohydrate, and even the glycogen of the muscle is not directly convertible into glucose.

The conversion of liver glycogen into glucose is believed to be due to a diastatic enzyme, *glycogenase*. In the extirpated liver, the conversion occurs very rapidly, but *in vivo* the conditions normally are apparently such that the amount of either substrate or enzyme available at any time is limited. It has been stated (Cori [10]) that, when insulin is lacking, the enzymic hydrolysis of liver glycogen is unchecked. This leads to the disappearance of the glycogen. A continuous supply of insulin by the pancreas is therefore essential for the preservation of hepatic glycogen. Opposed to this inhibition to glycogenolysis is the accelerating effect of epinephrine, the hormone of the adrenal medulla (p. 486). How epinephrine exerts this effect on the glycogenase in the hepatic cells of the living organism is not understood.

The fate of muscle glycogen will be considered in detail presently. Suffice it to state at this point that, whereas liver glycogen yields glucose, glycogen breakdown in muscle yields lactic acid, as first shown by Fletcher and Hopkins.[11] This has been particularly well brought out in an experiment by Simpson and Macleod.[12] They confined their studies to rabbit's liver and muscle. After freezing in liquid air these tissues were ground to a fine powder, and determinations were made of the changes in glycogen, glucose, and lactic acid. In the powdered liver preparations, as the glycogen disappeared, the free sugar increased, while the lactic acid content remained unchanged. The muscle preparations, on the contrary, showed no change in the free sugar, but an increase in lactic acid, as the glycogen diminished.

The administration of epinephrine causes a decrease in the glycogen content of muscle.

[10] *Physiol. Rev.*, **11**, 143 (1931). [11] *J. Physiol.*, **35**, 247 (1907).
[12] *Ibid.*, **64**, 255 (1927).

Blood Sugar. In the post-absorptive state the concentration of glucose is normally between 70 and 90 mg. per 100 cc., while during absorption of an ordinary meal it may rise temporarily to 120 or even 130 mg., returning to normal soon thereafter. The maintenance of the blood sugar within these narrow limits is due to the coordinated action of certain regulatory mechanisms. The blood sugar is, in fact, only one illustration of the relative stability of composition of the internal environment of the living organism, other examples being the maintenance of a constant *p*H, serum protein, sodium chloride, and calcium. The constancy in volume of the fluid matrix of the body and of the temperature also illustrates the condition of physiological stability, or *homeostasis*, the latter being a term that has been proposed by Cannon.[13]

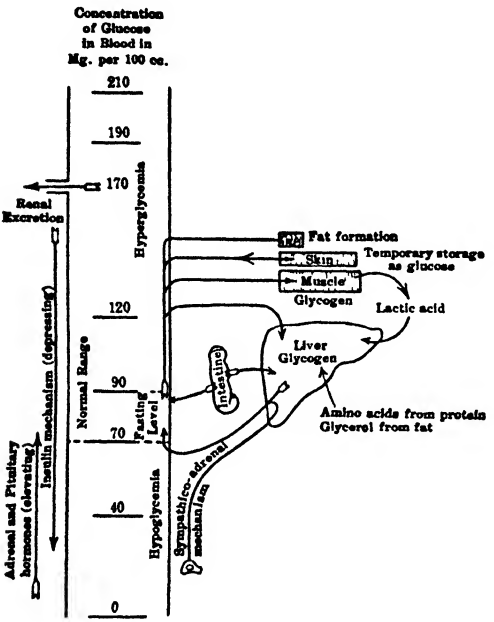


FIG. 30.—Schematic illustration of some of the factors which regulate the sugar concentration of the blood.

We may now consider the agencies which preserve the homeostasis of blood sugar. First it is to be observed that the fluctuations occurring normally represent the action of opposing forces depressing and elevating the sugar level. When the concentration of glucose in the blood is raised, it brings about a response which leads to its removal and storage in the liver, muscle, and skin. Storage in the skin is more transitory than in liver and muscle.[14] The generally held theory is that an increase in blood sugar stimulates the pancreas to secrete more insulin, which in turn promotes the deposition

[13] W. B. Cannon, *Physiol. Rev.*, **9**, 399 (1929); "The Wisdom of the Body," Norton, New York, 1932.

[14] H. C. Trimble and B. W. Carey, *J. Biol. Chem.*, **90**, 655 (1931).

Cannon distinguishes between two types of storage. One is storage by *inundation*; the other is storage by *segregation*. Storage of glucose in the skin is an example of the first type; it does not involve any chemical or physical change, and no special device is required either to store or to release it. It represents, in other words, the overflow from the blood stream. On the other hand, storage of glucose in the liver and muscle is by segregation; it depends on its conversion into glycogen, and, moreover, a special mechanism (hormonal, etc.) is involved both in its deposit and subsequent withdrawal.

of glycogen in liver and muscle, thereby lowering the concentration of glucose in the blood. Another conception (Soskin[9]) is that the liver itself responds to the influx of exogenous sugar by curtailing its output into the blood. In this way the exogenous sugar replaces the endogenous supply; utilization and storage rapidly return the blood sugar toward its normal level, whereupon the liver again takes up its secretory function.

Participation by the kidneys does not become prominent until the glucose concentration in the blood exceeds a certain value. This is usually given as 0.18 per cent (180 mg. per 100 cc.); but as this figure is based on older methods of blood-sugar analysis, the actual value is probably nearer 160 mg. The concentration of glucose in the blood at which there is an "overflow" into the urine is called the "renal threshold." Large amounts of glucose are excreted when the renal threshold is exceeded.[15]

These, in brief, are the principal factors concerned in maintaining the upper level of normal. In the regulation of the lower level, the adrenals and sympathetic nervous system and the liver are involved, and under certain conditions, at least, the so-called diabetogenic hormone of the pituitary and the hormone of the thyroid gland exert effects antagonistic to the action of insulin. The pancreatropic hormone of the pituitary stimulates the pancreas and is said to produce an insulin-like (blood-sugar lowering) effect. Any tendency to reduction below the normal is met by the release of glucose from the liver.

Departures outside the range of normal usually represent conditions of disturbed homeostasis. For example, in diabetes, there is believed to be a deficiency of insulin. As insulin exerts a depressing effect on blood sugar, a deficiency of the hormone, unless compensated by other factors, is associated with an increase in blood sugar. Antagonistic to the action of insulin is a sugar-raising hormone secreted by the anterior lobe of the pituitary. A lesion in this portion of the gland and an accompanying reduction in hormone may be associated with a tendency to low blood glucose; if the lesion of the pituitary occurs in a diabetic, the diabetes is ameliorated. On the other hand, if the production of insulin is excessive, as in certain tumors of the pancreas, a marked tendency to hypoglycemia (low blood sugar) may develop.

Adrenaline, the hormone of the adrenal medulla, is also an antagonist of insulin. In disease of the adrenals (Addison's disease) the production

[15] Not uncommonly individuals are encountered whose renal thresholds are low. In such cases marked glycosuria is observed even though there is little or no rise in the blood sugar. During pregnancy renal glycosuria of this type is not uncommon. In extreme cases, the permeability of the kidney to sugar appears to be so great that glucose is excreted even when the concentration in the blood is subnormal. Persons exhibiting this peculiarity may be quite normal otherwise. Finally, the renal threshold may be higher than 0.18 per cent. Especially in diabetics of long standing, the kidneys are often relatively impermeable to glucose even when the concentration in the blood reaches values as high as 0.25 per cent.

of adrenaline may be so reduced that there is not enough to oppose effectively the normal amount of insulin. This is another frequent cause of hypoglycemia.

Hypoglycemia occurs also in a number of conditions in which there seems to be a defect of the glycogen-to-glucose transformation in the liver. This defect is believed to be a factor in the rare disease, *hepatomegalia glycogenica* (von Gierke's disease).

Carbohydrate Tolerance. The capacity of the body to assimilate carbohydrates has long been the subject of clinical investigation. Before the advent of modern methods of blood analysis, this was measured by determining the amount of sugar which it was necessary to feed an individual before sugar appeared in the urine. A healthy person can tolerate 100 and even as much as 200 grams of glucose at a single dose without developing glycosuria. This was taken to indicate the efficiency of the tissues in removing the absorbed sugar from the blood. It is evident that individual variations in renal threshold may introduce an error in this method, for in the case of a high threshold no sugar would appear in the urine even though the accumulation of sugar in the blood were considerable, whereas in an individual with a low renal threshold marked glycosuria may develop even with a moderate increase in the sugar content of the blood.

With the introduction of simple methods for the quantitative determination of blood sugar, the changes in the concentration of this constituent have been taken as the basis for estimating carbohydrate tolerance. The blood is analyzed before and at certain intervals after giving a definite amount of glucose, usually 1.5 grams per kilogram of body weight. In normal subjects the blood sugar rises to a maximum during the first half-hour or hour and returns to approximately the original level by the end of the second hour. The analytical data obtained may be plotted on coordinate paper as ordinates, and the time intervals as abscissas. The maximum height of the curve, the time at which this maximum occurs, and the time required for the curve to return to normal are all taken into account in interpreting the results. Variations in the rate of absorption and the previous state of nutrition may have a modifying effect on the sugar-tolerance curve.

Carbohydrate tolerance is reduced in diabetes, in conditions where the liver is injured (phosphorus and chloroform poisoning), and to a less extent in acute infections and other pathological conditions. It is increased in hypopituitarism. In many individuals who develop hypoglycemia spontaneously it is difficult, or even impossible, to establish the underlying cause, but in others a definite association with hyperinsulinism (p. 473), or with Addison's disease can be demonstrated. In these conditions the glucose tolerance test often yields inconstant results, but as a rule a marked fall in concentration occurs at one, two, or more hours after taking the sugar. Inversion of the sugar tolerance curve,

like the abnormal curve in diabetes, is a clear indication of disturbed homeostasis with regard to blood sugar.

The First Stages in the Metabolism of Carbohydrate. A starting point in the discussion of carbohydrate metabolism is glycogen. Its decomposition to lactic acid is a familiar phenomenon, having been studied in frog muscle by Fletcher and Hopkins over thirty years ago. Offhand it may seem that this reaction should be a simple one; actually,

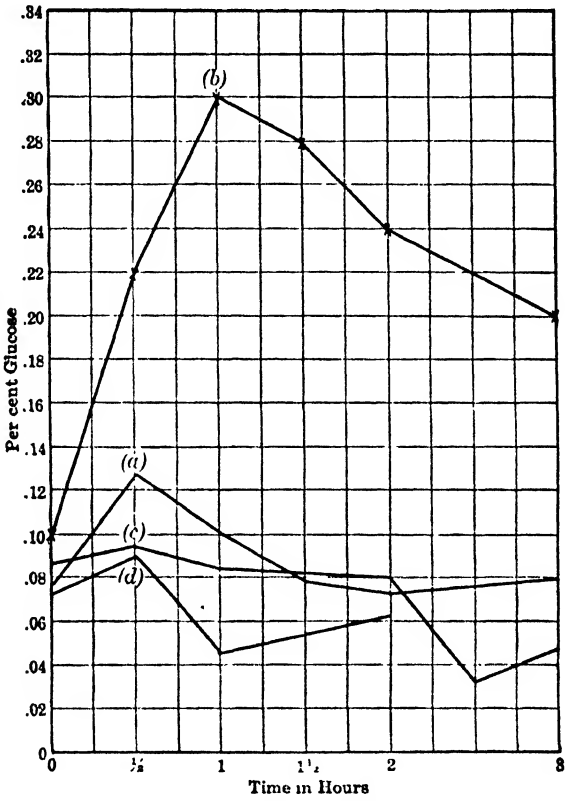


FIG. 31.—Glucose tolerance curves: (*a*) of a normal individual, (*b*) of a patient with diabetes, (*c*) of a person with tendency to spontaneous hypoglycemia due to hyperinsulinism (adenoma of the pancreas), (*d*) of a patient with Addison's disease

however, it has proved to be an exceedingly complex and elusive problem and has absorbed the efforts and ingenuity of some of the outstanding biochemists and physiologists of our time (Embden, Meyerhof, Parnas, Lohmann, Hill, Euler, and others) for nearly two decades. Every phase of the subject has been delved into, many intermediate products of the reaction have been isolated, and the participation of enzymes and coenzymes has been demonstrated. The information obtained in this way

has been carefully pieced together, and at times it seemed as though the fragments formed a coherent whole and that, at long last, the problem was practically solved; then would come the realization that one or more details had been overlooked, that the facts no longer fitted the theory that had just been so laboriously erected, and that therefore a new interpretation would have to be sought.

In view of this experience, and notwithstanding the important and tangible achievements of the last few years, it would be hazardous to assume that the problem is now solved and that our present conception is essentially a true and complete picture of the sequence of reactions involved in carbohydrate metabolism in muscle and other tissues. Though no such claim can be made; yet the reactions of carbohydrate combustion are of such immediate importance as to justify a brief résumé of the more general features of the subject.

If the formation of glucose were to represent an intermediate stage in the metabolism of muscle glycogen, the reaction

$$\text{Glucose} \xrightarrow{\text{(muscle extract)}} \text{lactic acid}$$

would occur very readily, in fact more readily than the reaction

$$\text{Glycogen} \xrightarrow{\text{(muscle extract)}} \text{lactic acid}$$

However, this is not the case. Lactic acid is produced from glucose or fructose much more slowly than from glycogen when added to muscle extract. Is it possible, therefore, that the conversion of glycogen to lactic acid does not include in its path the formation of a hexose sugar?

Muscle extracts contain an amylase or *glycogenase*, hence the inference that glycogen is first changed to glucose. This enzyme is somewhat more heat-stable than other enzymes, for, if muscle extracts are heated for a time to 38° C., they lose their capacity for transforming glycogen to lactic acid, but retain their power of forming reducing sugar. Such partially inactivated extracts are also capable of converting hexosediphosphoric acid into lactic acid. This suggests that the component affected must be one related to the esterification of glucose with phosphoric acid.

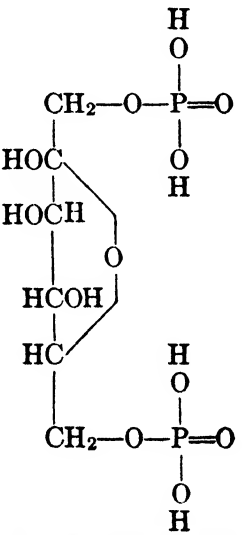
Meyerhof [16] found that the conversion of glucose to lactic acid by muscle extract could be greatly accelerated by the addition of an activator derived from yeast, to which the term "hexokinase" has been given. It is a water-soluble, thermolabile substance, and is presumably present in greater abundance in yeast than in muscle. Hexokinase seems to be concerned with the esterification of certain hexoses with orthophosphoric acid. It activates the formation of phosphoric acid

[16] O. Meyerhof, "Die chemischen Vorgänge in Muskel," Berlin, 1930.

esters of fructose about twice as rapidly as those of glucose, while exerting no effect on the esterification of galactose.

From these and other considerations it has been surmised that not glucose but one of its esters with phosphoric acid is the probable intermediate in the glycogen → lactic acid conversion.

Esters of Glucose, Fructose, etc., with Phosphoric Acid. *Hexosediphosphoric Acid.* Ever since Harden and Young [17] showed that phosphates accelerate the fermentation of sugars by yeast and discovered hexosediphosphoric acid in the products resulting from such fermentation, the relation of phosphates to carbohydrate metabolism has been the subject of intensive study. Hexosediphosphoric acid, according to Morgan and Robison,[18] and confirmed by Levene and Raymond,[19] is α-fructose-1 : 6-diphosphoric acid:

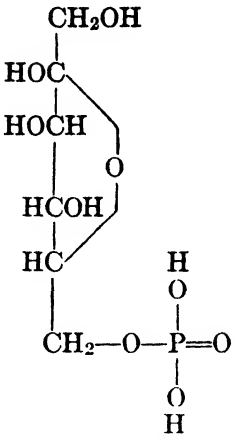


Fructosediphosphoric acid (Harden-Young ester)

Hexosediphosphoric acid ester may be designated as the Harden-Young ester, after its discoverers.

Hexosemonophosphoric Acid of Neuberg. The hexosediphosphoric acid of Harden-Young may be partly hydrolyzed to yield hexosemonophosphoric acid, as first shown by Neuberg.[20] This is believed to be α-fructose-6-monophosphoric acid and may be designated as the Neuberg ester.

[17] *Biochem. Z.*, **32**, 173 (1911); *Proc. Roy. Soc.* (*London*), *B*, **81**, 528 (1909).
[18] *Biochem. J.*, **22**, 1270 (1928).
[19] *J. Biol. Chem.*, **80**, 633 (1928).
[20] *Biochem. Z.*, **88**, 432 (1918).



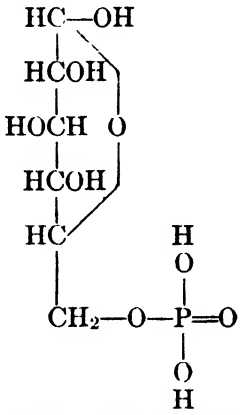
α-Fructose-6-monophosphoric acid (Neuberg ester)

The Robison-Embden Ester. The formation of hexosemonophosphoric acid, along with the diphosphoric ester of Harden and Young, was suspected by Harden and Robison [21] in 1914. In 1922 it was isolated by Robison [22] and found to differ from the Neuberg ester. Independently Embden and associates [23] discovered in muscle press juice an ester identical with the Harden-Young ester. Because it was thought to be the precursor of lactic acid it was named "lactacidogen." This designation was later transferred to a hexosemonophosphoric acid which Embden and Zimmermann [24] isolated from muscle. In time, evidence accumulated to show that this ester and the Robison ester were identical and that it was in reality an equilibrium mixture consisting of 70 per cent glucosemonophosphoric acid and 30 per cent fructosemonophosphoric acid. The equilibrium mixture is formed very rapidly from either the pure glucose or fructose ester, in the presence of an enzyme. The Robison-Embden ester is, moreover, a product of the enzymatic (muscle extract) hydrolysis of the Harden-Young ester, magnesium ions being required in the process. Although it was formerly considered that the Neuberg ester is exclusively a product of the acid hydrolysis of the Harden-Young ester, more recent work indicates its presence among the products of yeast fermentation (Robison).[25]

It should be noted in particular that the aldose and ketose monophosphates are interconvertible and that the reaction Harden-Young ester $\rightleftharpoons$ Robison-Embden ester is reversible.

[21] *Proc. Chem. Soc.*, **30**, 16 (1914).
[22] *Biochem J.*, **16**, 809 (1922)
[23] *Z. physiol. Chem.*, **93**, 124 (1914).
[24] *Ibid.*, **141**, 225 (1924).
[25] *Biochem. J.*, **26**, 2191 (1932).

The aldose component of the Robison-Embden ester is believed to be glucose-6-monophosphoric acid: [26]



Robison-Embden ester (glucose component)

Other Esters. Robison and Morgan [27] found that, in the fermentation of either glucose or fructose with dried yeast, trehalosemonophosphoric acid is one of the products formed. More recently Robison [25] has also discovered the presence of mannosemonophosphoric acid.

Lohmann [28] found two more esters, which at first appeared to be diphosphates, differing from the Harden-Young ester. These new forms accumulated when muscle "brei" was treated with either fluoride or iodoacetic acid. It was later shown by Embden [29] that under the conditions defined by Lohmann, glyceric-acid-monophosphoric acid (abbreviated phosphoglyceric acid) and α-glycerophosphoric acid were formed. The "Lohmann ester" may therefore be considered to be a mixture of the two compounds. The importance of these observations in contributing to our knowledge of carbohydrate metabolism will now be considered.

Relation of Hexosephosphates to Carbohydrate Metabolism. The discovery that phosphoglyceric and glycerophosphoric acids were products of enzymatic hydrolysis of fructosediphosphoric acid (Harden-Young ester) at once brought the latter compound into prominence in relation to carbohydrate metabolism. Notwithstanding its formation from glycogen in muscle extracts and in muscle under various experi-

[26] E. J. King, R. R. McLaughlin, and W. T. J. Morgan, *Biochem. J.*, **25**, 310 (1931); R. Robison and E. J. King, *ibid.*, **25**, 323 (1931); P. A. Levene and A. L. Raymond, *J. Biol. Chem.*, **89**, 479 (1930); **91**, 751 (1931).

The subject has been reviewed by Robison, "The Significance of Phosphoric Esters in Metabolism," New York University Press, 1932.

[27] *Biochem. J.*, **22**, 1277 (1928).

[28] *Naturwissenschaften*, **19**, 180 (1931); F. Lipmann and K. Lohmann, *Biochem. Z.*, **222**, 389 (1930).

[29] G. Embden, H. J. Deuticke, and G. Kraft, *Klin. Wochenschr.*, **12**, 213 (1933); *Z. physiol. Chem.*, **230**, 12, 29, 50 (1934).

mental conditions, it had not been isolated from normal muscle, and hence its significance was left in doubt. On the other hand, hexosemonophosphoric acid (Robison-Embden ester), because of its occurrence in muscle, was definitely linked with normal glucose metabolism, having been regarded by Meyerhof as the stabilization product of a much more labile ester, which as a rule did not accumulate, but was transformed *status nascendi* to lactic acid. The theory which grew out of Embden's latest discovery, without necessarily minimizing the importance of the equilibrium mixture of hexosemonophosphates, brought to the fore fructosediphosphoric acid as the probable intermediate product in the formation of lactic acid.

In 1933, shortly before his untimely death, Embden [29] presented a unified scheme of the reactions involved in the conversion of hexose to lactic acid. According to this scheme hexosediphosphoric acid (Harden-Young ester) is formed from hexose and hexosemonophosphoric acid reacting with phosphoric acid. Hexosediphosphoric acid is then decomposed to dihydroxyacetonephosphate and phosphoglycericaldehyde. By the process of dismutation these are in turn converted into glycerophosphoric and phosphoglyceric acids. The last forms pyruvic acid, which in turn reacts with glycerophosphoric acid to yield lactic acid and triosephosphoric (glycerophosphoric) acid. The last is ultimately utilized in the production of more lactic acid.

Embden's conception was promptly adopted by Meyerhof,[30] who with Kiessling [31] proposed an analogous scheme for the alcoholic fermentation of glucose by yeast.

One of the inconsistencies in Embden's system is that the reaction of pyruvic acid with glycerophosphoric acid requires more time than the entire sequence of reactions involved in the conversion of glycogen, or of hexosodiphosphate, to lactic acid. Difficulties of a similar kind affecting the rate of fermentation of phosphoglyceric and phosphopyruvic acids by yeast have also been encountered. Furthermore, a detailed study by Meyerhof and fellow-workers of certain reactions (glucose and pyruvic acid; glucose and phosphopyruvic acid; hexosediphosphate and pyruvic acid, etc.), disclosed that Embden's conception was untenable in its original form and that a revised formulation was necessary. Meyerhof's newer conception [32] will be referred to again presently.

Mention should now be made of Parnas, who has been very prominently connected with the subject and whose ideas of the sequence of the reactions associated with the conversion of glycogen to lactic acid seem to be especially illuminating. Before Parnas's view can be presented it is necessary to describe certain constituents of muscle which participate in these reactions.

[30] *Nature*, **132**, 337, 373 (1933).
[31] O. Meyerhof and W. Kiessling, *Biochem. Z.*, **264**, 40; **267**, 313 (1933).
[32] *Ibid.*, **281**, 249; **283**, 83 (1935).

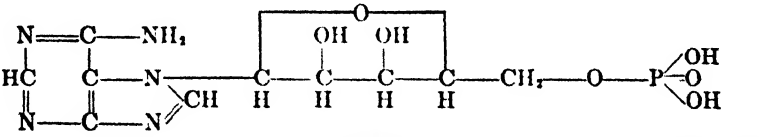
Adenosinetriphosphoric Acid. This compound is also described as adenosinetriphosphate, adenylic acid pyrophosphate, and adenylpyrophosphate.

In 1927 Embden and Zimmermann [33] discovered the presence in muscle of adenosine phosphate, or adenylic acid (p. 432).[34] At about the same time, Parnas and Mozolowski [35] discovered the formation of ammonia in muscle. It was subsequently shown that the ammonia was derived from the adenylic acid. Then followed the isolation by Lohmann [36] in 1929 of a compound which, on hydrolysis, in neutral solution or with acid, yielded adenylic acid and pyrophosphoric acid. This suggested that the adenylic acid was present in combination as pyrophosphate. Lohmann then showed that adenylic acid pyrophosphate (or adenylpyrophosphate) was essential to the breakdown of glycogen into lactic acid, its rôle in glycolysis being that of a coenzyme. The presence of magnesium ion was also found to be essential. Yeast likewise contains adenylpyrophosphate, this being presumably somewhat different from the compound in muscle. It plays an analogous rôle as a co-zymase in yeast fermentation, as shown by Euler and his associates.[37]

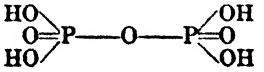
Phosphocreatine. Other names for this compound are creatinephosphoric acid and phosphagen. Its occurrence in muscle was reported in 1927 independently by Eggleton and Eggleton [38] and by Fiske and Subbarow [39] (p. 418).

[33] *Z. physiol. Chem.*, **167**, 137 (1927).

[34] The formula for adenosinetriphosphoric acid has not been definitely established. Adenylic acid may be represented as follows:



There is some difference of opinion as to whether adenylic acid exists as the pyrophosphate or as a triphosphate. Pyrophosphoric acid ($H_4P_2O_7$) has the following formula:



[35] *Biochem. Z.*, **184**, 399 (1927).

[36] *Naturwissenschaften*, **17**, 624 (1929).

[37] *Z. physiol. Chem.*, **165**, 140 (1927); **168**, 177 (1927); **177**, 237 (1928); **184**, 163 (1929). Yeast contains another coenzyme (co-zymase). On hydrolysis it yields adenine and nicotinamide. See F. Schlenck and H. V. Euler, *Naturwissenschaften*, **24**, 794 (1936); K. Myrbäck, *Z. physiol. Chem.*, **241**, 223 (1936).

[38] *Biochem. J.*, **21**, 190 (1927); *J. Physiol.*, **63**, 155 (1927).

[39] *Science*, **65**, 401 (1927); *J. Biol. Chem.*, **81**, 629 (1929).

Soon after the discovery of phosphocreatine, it was shown by Meyerhof [40] that its hydrolysis, whether by acid or enzyme, is accompanied by the liberation of heat (11,000 to 12,500 calories per mole). Then followed the observation of Nachmansohn [41] that in muscular activity the phosphocreatine first broken down is rapidly restored even in the absence of oxygen. Two years later Lundsgaard [42] described an experiment in which it was shown that frog's muscle poisoned with iodoacetic acid may contract, but that no lactic acid is formed in the process. This was an astounding discovery, as it was contrary to the prevailing conception that the energy of muscular contraction is derived from the formation of lactic acid. The theory was therefore advanced that the hydrolysis of phosphocreatine is the primary change and supplies the energy in the normal contraction of muscle. This view has been considerably weakened by the work of Lohmann,[43] which showed that the liberation of phosphate from adenosinetriphosphoric acid precedes the hydrolysis of phosphocreatine. The phosphate liberated in the last reaction may combine with adenylic acid to restore the adenosinetriphosphoric acid.

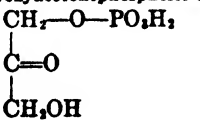
In the following scheme of Parnas [44] the relation of adenosinetriphosphoric acid and of phosphocreatine to the metabolism of glycogen is brought out. It should be observed that Parnas believes that the formation of fructosediphosphate from glycogen is direct and does not involve the intermediate production of free hexose.

[40] *Biochem. Z.*, **191**, 106 (1927).
[41] *Ibid.*, **196**, 73 (1928).
[42] *Ibid.*, **217**, 162; **220**, 1, 8; **227**, 51 (1930); **230**, 10; **233**, 322 (1931).
[43] *Naturwissenschaften.*, **22**, 409 (1934).
[44] *Klin. Wochschr.*, **14**, 1017 (1935).

Formulas of Intermediate Compounds included in Parnas's Scheme

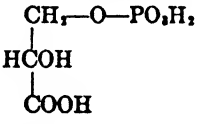
Fructosediphosphate, p. 323.

Phosphodihydroxyacetone (dihydroxyacetonephosphoric acid)

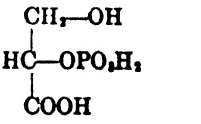


Inosinic acid, p. 431.

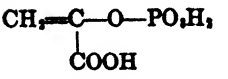
β-Phosphoglyceric acid



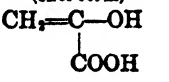
α-Phosphoglyceric acid



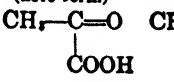
Phosphopyruvic acid



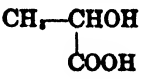
Pyruvic acid (enol form)



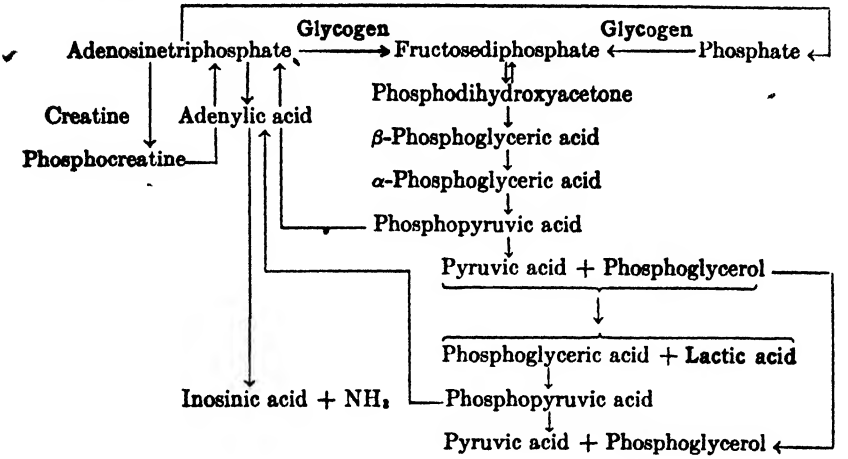
Pyruvic acid (keto form)



Lactic acid



OUTLINE OF LACTIC ACID PRODUCTION IN MUSCLE, ACCORDING TO PARNAS[44]



Meyerhof and Kiessling's scheme[45] of lactic acid formation differs from that of Parnas in certain particulars, as indicated by the following sequence of reactions:

(1) 2 **Hexose** + 2 adenylpyrophosphate = 2 hexosediphosphate + 2 adenylic acid
(2) 2 Hexosediphosphate $\rightleftharpoons$ 4 phosphohydroxyacetone $\rightleftharpoons$ (4 phosphoglyceric aldehyde) $\rightarrow$ 2 α-phosphoglycerol + 2 phosphoglyceric acid
(3) 2 Phosphoglyceric acid $\rightleftharpoons$ 2 α-phosphoglyceric acid $\rightleftharpoons$ phosphopyruvic acid
(4) 1 Hexose + 2 phosphopyruvic acid $\rightarrow$ hexosediphosphate + 2 pyruvic acid
(5) 1 Hexose + $2H_3PO_4$ + 2 Pyruvic acid $\rightarrow$ { "Primary ester" + Pyruvic acid $\rightarrow$

2 Triosephosphate + 2 Pyruvic acid $\rightarrow$ { 2 Phosphoglyceric acid + 2 Lactic acid

(5a) Hexosediphosphate + 2 dihydroxyacetonephosphate $\rightarrow$ 2 phosphoglyceric acid + 2 pyruvic acid $\rightarrow$ 2 pyruvic acid + 2 lactic acid

Aside from other differences, it is to be observed that in Parnas's scheme glycogen is the starting point, whereas in Meyerhof's it is hexose.

Alcoholic Fermentation. To acquire a comprehensive view of the subject, the student must turn to the original literature, from which he will discover the experimental basis for the present position. In all probability this will undergo further modification in the future.

In 1933 it seemed to Meyerhof that, through the stage of pyruvic acid, Embden's scheme applied to alcoholic fermentation, the last stages consisting in the conversion of pyruvic acid to acetaldehyde, and the reaction of the latter with triosephosphoric acid to form ethyl alcohol

[44] *Biochem. Z.*, **283**, 83 (1936).

and phosphoglyceric acid. However, during the two years that followed, an exhaustive study of the dynamics of the individual reactions led to the adoption in 1935 of a new scheme of reactions, analogous to the one for muscle lactic acid. Finally as a result of certain observations in Warburg's laboratory, Meyerhof [46] was led to a further study of the intermediate reactions in alcoholic fermentation and on the basis of new data was again obliged to modify his former conception. The scheme recently proposed by Meyerhof, Kiessling, and Schultz is as follows:

1 *M* **Hexosediphosphate** $\rightleftharpoons$ 1 *M* phosphoglyceric aldehyde + 1 *M* dihydroxyacetonephosphate = 2 *M* triosephosphate

2 *M* Triosephosphate + 2 *M* glucose + 2 *M* H_3PO_4 + 2 *M* acetaldehyde = 2 *M* β-phosphoglyceric acid + 2 *M* hexosemonophosphate + **2 *M* Alcohol**

2 *M* β-Phosphoglyceric acid $\rightleftharpoons$ 2 *M* α-phosphoglyceric acid $\rightleftharpoons$ 2 *M* phosphopyruvic acid + $2H_2O$

2 *M* Hexosemonophosphate + 2 *M* phosphopyruvic acid = 2 *M* pyruvic acid + **2 *M* Hexosediphosphate**

2 *M* Pyruvic acid = $2CO_2$ + 2*M* acetaldehyde

It has been shown recently by Lohmann and Schuster [47] that the coenzyme of carboxylase (cocarboxylase) participating in the oxidation of pyruvic acid is the diphosphoric acid ester of vitamin B_1, (p. 588). A similar mechanism is involved in the oxidation of pyruvic acid in brain and possibly in other tissues.

Fate of Lactic Acid Formed in Muscle. The lactic acid formed from carbohydrate during muscular activity is disposed of principally in three ways:

1. Oxidation to CO_2 and H_2O.
2. Conversion to glycogen in the muscle.
3. Diffusion into the blood, removal by the liver of the portion not excreted by the kidneys, and its synthesis to glycogen.

Oxidation. We have seen (p. 311) that lactic acid is oxidized in the presence of a muscle enzyme and a coenzyme. The product, pyruvic acid, is in turn oxidized to CO_2 and H_2O, but there is still some uncertainty as to the intermediate reactions. It has been disclosed, however, that in alcoholic fermentation, the oxidation of pyruvic acid depends on the presence in the yeast of vitamin B_1 (as its diphosphoric acid ester). Vitamin B_1 also appears to be the coenzyme in the system involved in the oxidation of pyruvate in brain tissue, and possibly in other tissues as well. In the absence of the coenzyme, pyruvate fails to be oxidized and therefore accumulates, and in turn this inhibits almost completely the oxidation of lactic acid.

[46] O. Meyerhof and W. Kiessling, *Biochem. Z.*, **281**, 249 (1935); *Ibid.*, **292**, 25 (1937).

[47] K. Lohmann and Ph. Schuster, *ibid.*, **294**, 183 (1937).

Oxidation of lactic acid provides most of the energy for muscular activity. Lohmann [48] has estimated that this reaction provides 20 to 40 times as much energy as all three reactions which occur anaerobically, namely hydrolysis of adenosinetriphosphoric acid, hydrolysis of phosphocreatine, and conversion of glycogen to lactic acid. In passing, it may be mentioned that when muscle contracts isometrically, i.e., without shortening, all the energy which it expends appears as heat, but if muscle shortens and does work in lifting a weight, a portion of the energy, usually about 25 per cent, is expended in doing mechanical work.

Conversion to Glycogen in Muscle. Meyerhof [49] found that in isolated frog muscle, a major part (four-fifths) of the lactic acid formed during contraction is resynthesized to glycogen during the stage of recovery. This is regarded as one of the important changes occurring during oxidative recovery which restore the muscle to its original status.

Meyerhof's conclusion, in the main, has been widely accepted. His observations have been confirmed for isolated rabbit and beef muscle by Boyland,[50] but contrary results have been obtained by Sacks and Sacks [51] in a study of the changes in the gastrocnemius of the intact mammal (rabbit). They have reported that the entire amount of lactic acid formed during anaerobic activity diffuses into the blood stream during recovery and that glycogen is not formed in muscle from any of the lactic thus produced. If these contentions are adequately confirmed it would mean that the "Meyerhof cycle" is of no significance in recovering mammalian muscle.

Lactic Acid Production, Diffusion into Blood, and Conversion into Glycogen in the Liver. Even in a state of rest, the blood contains lactic acid, which has been estimated to vary between 5 and 20 mg. per 100 cc. of blood.[52] In moderate exercise the blood lactic acid increases. For example, walking at a rate of 3.5 miles per hour was found by Hill, Long, and Lupton [53] to cause the lactic acid content of the blood to increase from 20.9 mg. (the concentration before the period of exercise) to 36.6 mg. per 100 cc. Somewhat more strenuous exercise, namely walking at the rate of 4.1 miles per hour, produced an increase from 21.4 mg., the initial value, to 58.9 mg. per 100 cc. Quite obviously, during muscular exercise, there is increased production of lactic acid and a somewhat greater amount escapes into the blood. If the exercise is moderate, the lactic acid, after reaching a certain level, does not continue to accumulate either in the muscles or in the blood. There is sufficient respiratory stimulation to provide an adequate extra supply of oxygen to keep pace

[48] Oppenheimer's "Handb. d. Biochemie," 3d Supplement, p. 370, 1935.

[49] *Arch. ges. Physiol.*, **185**, 11 (1920); compare with A. V. Hill, *Physiol. Rev.*, **2**, 329 (1922).

[50] *Biochem. Z.*, **237**, 418 (1931).

[51] *Am. J. Physiol.*, **112**, 565 (1935).

[52] Compare A. V. Bock, D. B. Dill, and H. T. Edwards, *J. Clin. Investigation*, **11**, 775 (1932).

[53] *Proc. Roy. Soc.* (*London*), *B*, **96**, 438; **97**, 84, 155 (1924).

with the increased lactic acid formation and in this way a balanced condition is reached which has been referred to as the *steady state*. This means that there is a steady rate of oxygen utilization, and that the lactic acid content of the muscle, though above normal, is nevertheless maintained at a constant level.

The situation is different when the exercise is more strenuous in character. First, with regard to the lactic acid in the blood, there is a marked increase. In an experiment of Hill and his associates, the subject ran in a standing position for 4 minutes (breathing pure oxygen). At rest, the lactic acid content was 20 mg. per 100 cc., whereas immediately after the exercise it was 86 mg. In another experiment, the subject ran in place at 239 steps per minute for 9.5 minutes, breathing air. The lactic acid rose from 8.5 to 204 mg. per 100 cc. A similar effect was observed by Barr, Himwich, and Green.[54] In a series of experiments they subjected a number of individuals to approximately 3500 kilogrammeters of work, performed in a period of 3.5 minutes, and determined, among other things, the change in lactic acid in the blood. Invariably there was an increase. The difference between the lactic acid concentration at rest and at the end of the exercise ranged between 31.7 mg. and 85.8 mg.

The essential difference between moderate and very strenuous exercise is that in the latter there is a considerable accumulation of lactic acid in the muscle. This is because the supply of oxygen, after reaching a limiting value, cannot be increased any further and does not keep pace with the lactic acid production. When light or moderate exercise is stopped, there is a prompt return to normal of the gaseous exchange, and the lactic acid concentration in the blood begins to fall. Not so when violent exercise is suddenly terminated. For some minutes thereafter the lactic acid content of the blood continues to increase (see for example Barr and Himwich [55]), before the drop sets in, and the oxygen utilization continues at a high level for a considerable period. During strenuous exercise when the oxygen supply is inadequate, the tissues go into "oxygen debt," and a long period of recovery, in which the oxygen utilization continues at a high level, is required before the debt is paid. Thus, in the experiments of Hill, Long, and Lupton, a subject, after running 3 meters per second for 5 minutes, took 9.5 minutes to recover (i.e., before his oxygen intake returned to normal) and his oxygen debt was 1.7 liters. After running in place for 20 seconds as violently as possible, he went into debt 5.5 liters of oxygen and took 14 minutes to recover. A quarter of a mile run, followed by severe gymnastic exercise, resulted in an oxygen debt of 12.4 liters, the subject taking 44 minutes to recover.

Margaria, Edwards, and Dill [56] have presented evidence to show

[54] *J. Biol. Chem.*, **55**, 495 (1923).
[55] *Ibid.*, **55**, 539 (1923).
[56] *Am. J. Physiol.*, **106**, 689 (1933).

that an oxygen debt is incurred in muscular activity even before there is evidence of a lactic acid increase. This so-called *alactacid* oxygen debt has been related to the oxidation of substances (ordinary fuel) furnishing the energy for the resynthesis of phosphagen.

Now, what we are primarily interested in here is the fate of the lactic acid which escapes into the blood, for, it is seen, this occurs in a measure even in the resting condition, but is much increased during exercise. A small portion of the lactic acid is excreted in the urine, and the amount lost in this way may be considerable during violent exercise; another portion may be removed by other tissues (brain?) and oxidized; but perhaps the largest proportion is removed by the liver, where it is resynthesized into glycogen.

Partial evidence of this has been submitted by Himwich, Koskoff, and Nahum,[57] who analyzed blood removed simultaneously from the femoral artery, femoral vein, portal vein, hepatic artery, and the hepatic vein of dogs after varying periods of exercise. They found that blood going to muscle (femoral artery) contained less lactic acid than blood leaving it (femoral vein). On the other hand, blood entering the liver (hepatic artery, portal vein) contained more lactic acid than blood leaving it (hepatic vein). As an illustration of the conspicuous differences are the following figures for lactic acid (milligrams per 100 cc. of blood) obtained in an experiment in which the dog had been exercised for 5 minutes: hepatic artery 65, portal vein 42, hepatic vein 24.

It is of interest that the relations were reversed in regard to glucose. The blood of the femoral artery contained more glucose than blood of the femoral vein; the blood of the hepatic artery less than the blood of the hepatic vein.

Bott and Wilson [58] have shown that lactic acid does not accumulate in the liver when it disappears from the blood after exercise. The lactic-acid concentration in the liver rises and falls with changes in the blood, but usually remains considerably below the blood level.

Parnas and Baer [59] long ago demonstrated glycogen synthesis in the turtle liver, perfused with sodium lactate. Izume and Lewis [60] observed glycogen deposition in the liver of fasting rabbits injected subcutaneously with sodium lactate, and more recently Cori and Cori,[61] working with rats, found that if sodium *d*-lactate is fed by mouth or injected subcutaneously, glycogen is deposited in the liver. Sodium *l*-lactate, though absorbed from the intestine at the same rate as the *d*-isomer, formed hardly any liver glycogen. Cori and Cori state that of the *d*-lactate absorbed in three hours, 40–95 per cent was retained as liver

[57] *J. Biol. Chem.*, **85**, 571 (1930).
[58] *J. Biol. Chem.*, **109**, 463 (1935).
[59] *Biochem. Z.*, **41**, 414 (1912).
[60] *J. Biol. Chem.*, **71**, 51 (1926–27).
[61] *Am. J. Physiol.*, **81**, 389 (1929); see also C. F. Cori, *Physiol. Rev.*, **11**, 143 (1932); *Harvey Lectures*, 1927–28, p. 76 (1929).

glycogen and none was excreted in the urine, whereas 30 per cent of the *l*-lactate absorbed was recovered in the urine. Contrary results were obtained by Abramson, Eggleton, and Eggleton,[62] who failed to demonstrate the synthesis of glycogen from racemic sodium lactate in the liver of dogs under amytal anesthesia.

Previously fasted animals subjected to prolonged and strenuous exercise may show marked depletion of body glycogen without any change in liver glycogen, the latter being readily synthesized from lactic acid. As shown by the experiments of Long and Grant,[63] the restoration of body (muscle) glycogen, under these circumstances, occurs much more slowly, requiring about 12 hours for its completion.

During exercise in the undernourished animal the increase in fixed acids (chiefly lactic) in the blood is in excess of the amounts present in normally fed, exercised animals. Moreover, the return to the initial state is a much slower process, suggesting, according to Schlutz, Hastings, and Morse,[64] a disturbance in the mechanism for delivering oxygen to, and removing metabolic products from, the tissues.[65]

Evidence that muscle glycogen is not converted into glucose is given by the work of Bollman, Mann, and Magath.[66] They found that, when the liver is removed, the blood sugar rapidly falls to very low values. As the muscle contains considerable amounts of glycogen and as this does not prevent the hypoglycemia and does not disappear to any great extent, it is concluded that muscle glycogen is not readily converted into glucose.

It is well known that epinephrine, ether anesthesia, and asphyxia produce hyperglycemia. But, as has been shown by Soskin,[67] if the abdominal viscera, including the liver, are removed, hyperglycemia does not develop under these conditions despite the fact that there is glycogen in the muscles.

Weighing the foregoing evidence leads to the conclusion that muscle glycogen gives rise to lactic acid, but not to glucose; that the lactic acid thus formed, which reaches the liver, is converted to glycogen. A glucose molecule can therefore go through a complete cycle in the body; it can in turn be liver glycogen, blood sugar, muscle glycogen, blood lactic acid,

[62] *J. Biol. Chem.*, **75**, 763 (1927).

[63] *Ibid.*, **89**, 553 (1930).

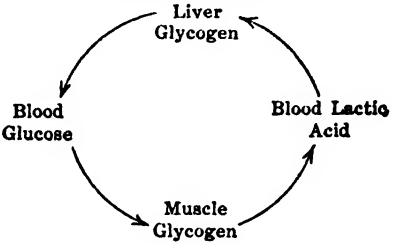
[64] *Am. J. Physiol.*, **104**, 669 (1936).

[65] The altered capacity of the tissues to oxidize carbohydrate, as a result of starvation, has been studied by Dann and Chambers (*J. Biol. Chem.*, **89**, 675 [1930]; **95**, 413 [1932]; **100**, 493 [1933]). They found that the oxidation of glucose fed to dogs that had been fasted for a period of three weeks is completely suppressed for about four hours following its ingestion. Carbohydrate metabolism was not restored to normal if only glucose were fed, suggesting that a fundamental change had been produced in the sugar-oxidizing mechanism, the return of which to normal depends on factors not yet determined.

[66] *Am. J. Physiol.*, **74**, 238 (1925).

[67] *Ibid.*, **81**, 382 (1927).

and again liver glycogen. This cycle, based on the work of Himwich [67] and Cori and Cori,[61] may be represented as in the diagram.



Carbohydrate Metabolism of Brain and Nerve.[68] Himwich and Nahum [69] studied the respiratory metabolism of the brain of dogs and concluded from their results that carbohydrate was used exclusively as the source of energy. They found, moreover, that the venous blood from the brain contained less dextrose and lactic acid than the blood going to the brain, indicating that both these substances were oxidized. Even in diabetes, the utilization of carbohydrate seemed unimpaired.

Differing from the respiratory quotient of brain, which is close to 1.0, is the respiratory quotient of resting nerve, which varies between 0.75 and 0.80. Such values point to the utilization of a mixture of fat, protein, and carbohydrate. During activity, the respiratory quotient of nerve rises. If the respiratory quotient of the extra metabolism is computed, it is found to vary between 0.95 and 1.0. This corresponds to the values which would be obtained if only carbohydrate were being oxidized, or if protein were being utilized in such a way as to form ammonia, rather than urea, as the end-product of the nitrogenous metabolism.

The influence of narcotics in depressing the oxygen consumption of brain has been studied by Quastel and Wheatley.[70]

The reactions of carbohydrate metabolism in brain closely resemble those in muscle, but it is improbable that the sequence of reactions is identical in the two tissues. One apparent difference is that glucose, and not glycogen, is the principal substrate in brain metabolism. That the processes are not, however, very divergent is shown by the presence of the same chemical components, including enzymes and coenzymes, which participate in muscle metabolism. Oxidation of pyruvic acid, an intermediate in carbohydrate metabolism, requires the presence of vitamin B_1 (p. 588), which acts as the coenzyme of carboxylase (cocarboxylase).

Owing to the instability of certain of the brain constituents, their isolation and quantitative estimation requires freezing of the tissue *in situ* with liquid air, a procedure that has been put to good use recently by

[68] The subject has been reviewed recently by I. H. Page, "Chemistry of the Brain," C. C. Thomas, Springfield, 1937.

[69] *Am. J. Physiol.*, **90**, 389 (1929); **101**, 446 (1932).

[70] *Proc. Roy. Soc.* (*London*), *B*, **112**, 60 (1932); *Biochem. J.*, **28**, 1521 (1934).

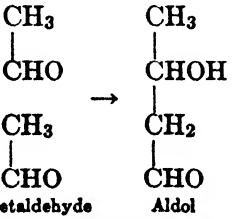
Kerr [71] and others. Kerr has determined that somewhat less than half of the creatine in brain exists as phosphocreatine; the highest value obtained was equivalent to 59 mg. of bound creatine per 100 grams of cat brain. He also succeeded in isolating phosphocreatine from cat brain as the crystalline calcium salt.

The glycogen content is comparatively low, being in the neighborhood of 100 mg. per 100 grams of brain tissue. Kerr and Ghantus found that it was not influenced by fasting, overfeeding, glucose administration, phlorhizin poisoning, or pancreatectomy. However, overdosage with insulin caused a marked decrease (50 to 70 per cent in some experiments) of the brain glycogen of dogs and rabbits.

Glucose is present in lower concentration than in the blood, amounting to 35–75 mg. per 100 grams of rabbit brain and 45–86 mg. in dog brain. Higher values accompany hyperglycemia; lower values occur when the blood sugar is reduced, except in extreme insulin hypoglycemia, when the concentration in the brain may be somewhat above that in the blood. During anaerobic incubation, the amount of lactic acid formed corresponds to the loss of free sugar (which disappears within 3 to 5 minutes) and glycogen (80 to 85 per cent of which is lost within 15 minutes). Avery, Kerr, and Ghantus [71] have shown that the concentration of lactic acid in brain is of the same order of magnitude as the concentration ordinarily found in muscle and blood of resting individuals. For cat's brain they obtained an average of 15.3 mg. and for dog's brain 22.3 mg. per 100 grams of tissue.

The major trends in the chemical investigation of the nervous system have been ably summarized by Gerard [72] in a recent review.

The Conversion of Carbohydrate to Fat.[73] The synthesis of fat from carbohydrate in the animal organism is an established fact and a matter of common knowledge and experience. It involves the formation from glucose of both glycerol and fatty acids. From their chemical relationship, the origin of glycerol from glyceric aldehyde, or some other 3-carbon intermediate of carbohydrate metabolism, seems probable. The origin of the fatty acids, on the contrary, is a somewhat more debatable point. These are probably formed from acetic aldehyde by aldol condensation, as originally suggested by Nencki. This may be represented as follows:



2 Acetaldehyde Aldol

[71] *J. Biol. Chem.*, **110**, 625, 637 (1935); S. E. Kerr and M. Ghantus, **116**, 9 (1936); **117**, 217 (1937).

[72] *Ann. Rev. Biochem.*, **6**, 419 (1937); see also *Physiol. Rev.*, **12**, 469 (1932).

[73] The subject has been comprehensively reviewed by D. Rapport, "The Interconversion of the Major Foodstuffs," *Physiol. Rev.*, **10**, 349 (1930).

Through the reduction of the hydroxyl group and oxidation of the aldehyde group, butyric acid would be obtained. Or by repeated aldol condensation, the higher fatty acid homologues would be derived.

This general view has been adopted by Leathes and Raper,[74] who consider that the process may involve the primary synthesis of longer chains of unsaturated fatty acids, as indicated by the following formulas:

$$4CH_3 \cdot CHO \rightarrow CH_3 \cdot CH : CH \cdot CH : CH \cdot CH : CH \cdot CHO$$

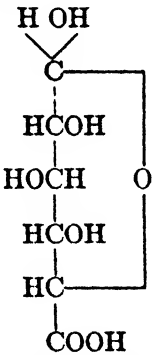
Reduction followed by oxidation of the aldehyde group would yield an 8-carbon saturated fatty acid, $CH_3(CH_2)_6COOH$. It is assumed that the higher fatty acids may be formed in a similar manner.

Smedley and Lubrzynska[75] have suggested that the mechanism of fatty acid synthesis from carbohydrate may involve the condensation of acetic aldehyde with pyruvic acid, $CH_3 \cdot CO \cdot COOH$, derived by the oxidation of methylglyoxal (pyruvic aldehyde), yielding a ketonic acid which is converted by the splitting off of carbon dioxide into an aldehyde having one carbon atom less than the ketonic acid. The aldehyde then condenses with another molecule of pyruvic acid and again gives off a molecule of carbon dioxide. By the repetition of this process, long carbon chains may be built up. This type of synthesis has been observed *in vitro* in the case of butyl aldehyde and pyruvic acid. When the fatty acid chain is built up, it very likely undergoes a certain amount of oxidation yielding intermediate compounds containing unsaturated linkages. One stage in the synthesis may be represented as follows:

$$CH_3CHO + CH_3CO \cdot COOH \rightarrow CH_3CH : CH \cdot CO \cdot COOH + H_2O$$

$$CH_3CH : CH \cdot CO \cdot COOH \rightarrow CH_3CH : CH \cdot CHO + CO_2$$

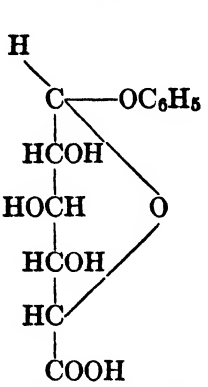
Glycuronic Acid. The formation of this compound in the animal organism reveals a path of glucose oxidation unlike any considered hitherto. Glycuronic acid was first isolated from the urine of animals that had been fed camphor, but it has been detected since, though in small amounts, in normal urine.



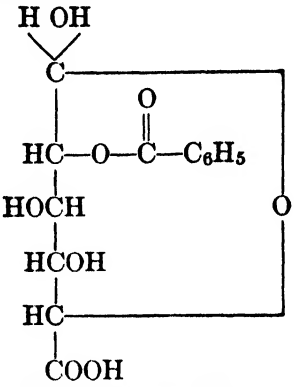
Glycuronic acid

[74] J. B. Leathes and H. S. Raper, "The Fats," London, 1925.
[75] *Biochem. J.*, 7, 364, 375 (1913).

Glycuronic acid does not exist in the urine in the free form, but in combination, chiefly with compounds of the aromatic series. Two kinds of linkage occur—the glucosidic type, as in phenol-, or menthol-glycuronic acid; and the ester type, as in glycuronic acid monobenzoate.

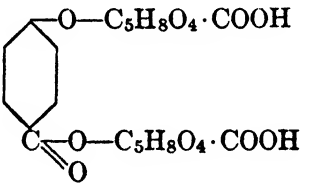


Phenylglycuronate



Glycuronic acid monobenzoate

When hydroxybenzoic acid is fed to dogs, it is conjugated with two molecules of glycuronic acid, forming an ester linkage with one and a glucoside linkage with the other, as indicated by the following formula:



Administered in small amounts (about 3 grams), glycuronic acid disappears completely. Given in larger amounts, a portion is recovered in the urine. Although in general it appears that the organism possesses only a limited capacity to oxidize this substance, it is interesting to note that in dogs mentholglycuronic acid is oxidized almost completely. In man, the administration of glycuronic acid monobenzoate leads to an excretion of hippuric acid (benzoylglycine), suggesting the cleavage and possible oxidation of the glycuronic acid residue.

In the past, glycuronic acid was considered chiefly from the standpoint of a detoxicating agent, it being assumed that the process of conjugation resulted in rendering a toxic substance less toxic, or non-toxic. Quick,[76] to whose work we owe much of our present knowledge of glycuronic acid metabolism, has recently pointed out the interesting fact

[76] A. J. Quick, *J. Biol. Chem.*, **61**, 667, 679 (1924); **67**, 477; **70**, 59, 397 (1926); **74**, 331 (1927); **80**, 535 (1928); **92**, 65 (1931); **95**, 189; **96**, 83; **97**, 403 (1932); **99**, 119 (1932–33); **100**, 441 (1933); compare also J. Pryde and R. T. Williams, *Biochem. J.*, **28**, 131 (1934), and A. J. Quick, *ibid.*, p. 403.

that conjugation with glycuronic acid yields products which are much stronger acids than the original unconjugated substances. He has therefore suggested that a fundamental factor in conjugation is the conversion of a weak acid, which the body apparently cannot excrete, to a strong acid which it can eliminate through the kidney.

It is conceivable that the physiological significance of glycuronic acid is not restricted to its function in forming conjugation products. As has been emphasized by Quick, it stands to reason that any substance which the human organism can synthesize at the rate of nearly 1 gram per hour cannot be ignored without incurring the danger of overlooking perhaps an important metabolic process.[77]

Of interest is the observation of increased urinary excretion of *l*-xyloketose by pentosuric individuals after feeding glycuronic acid, or glycurorgenic drugs, such as antipyrine, pyramidone, menthol (Enklewitz and Lasker[78]).

Fate of Other Carbohydrates. The conversion in the body of glucose into galactose is indicated by the synthesis of lactose in the mammary glands of lactating mammals. Pentoses (*d*-ribose and *d*-ribodesose) enter into the synthesis of nucleic acids and of closely related constituents of cells. Galactosamine has been reported present along with glucosamine in various protein combinations. Moreover, galactose exists in combination with lipids in nervous tissue (galactolipids or cerebrosides), and together with glucose in the mucoproteins. Glucose and mannose are also known to occur in other proteins.

Cartilage, bone, tendons, and fascia contain chondroitin, an amino polysaccharide, conjugated with sulfuric acid.

These are among the better-known tissue constituents containing carbohydrate in combination. Their synthesis in the body must therefore be considered as part of the carbohydrate metabolism. What becomes of these substances in catabolism is uncertain. Perhaps a certain amount of these carbohydrates is reconverted into glucose and shares its fate in metabolism. There is also a strong likelihood that the small amounts of pentose, galactosamine, and glucosamine, etc., which may be formed in tissue catabolism are excreted in the urine unchanged.

Glycosuria. The presence of sugar in the urine is termed glycosuria (glucosuria) or mellituria. It may be entirely physiological, but is often a sympton of diabetes, a disease in which the metabolism of carbohydrates is impaired.

When sugar is found in the urine, it is nearly always glucose. Rarely,

[77] From pregnancy urine, S. L. Cohen and G. F. Marrian, (*Biochem. J.*, **30**, 57 [1936]), have isolated a compound, the composition of which corresponds to oestriol-glycuronic acid. E. M. Venning and J. S. L. Browne (*Proc. Soc. Exptl. Biol. Med.*, **34**, 792 [1936]) have reported the isolation of pregnandiolglycuronide from pregnancy urine. The significance of the occurrence of the sex hormones in this form of combination remains to be determined.

[78] *J. Biol. Chem.*, **110**, 443 (1935).

fructose [79] and pentose (*l*-xyloketose) [80] may be present. During the last stages of pregnancy, particularly during the last few days before delivery, as well as during lactation, excretion of lactose is not uncommon. Following the injection or rapid absorption of disaccharides (sucrose, maltose), these may appear in the urine.

Sugar appears in the urine either when its concentration in the blood is high and exceeds the renal threshold, or when the kidneys are unusually permeable to this substance. The concentration of glucose in the blood may increase as a result of insufficient glycogenesis or because of an excessive amount of glycogenolysis. When large amounts of carbohydrate are being absorbed and the rate of conversion into glycogen does not keep pace with the rate of absorption, there is a piling up of sugar in the blood (hyperglycemia) and the excretion of part of it in the urine. When the appearance of sugar in the urine is due to the ingestion of excessive amounts of carbohydrate, the condition is termed *alimentary glycosuria*. This is purely a physiological phenomenon and may be produced in normal individuals.

In a considerable proportion of normal individuals there is a tendency to glycosuria in the afternoon, as recently shown by Harding, Selby, and Armstrong.[81] It has been assumed by these authors that "afternoon glycosuria" may be due to a lowering in the renal threshold at that time of the day.

Deficient glycogenesis occurs in conditions of acidosis and of liver injury (alcoholism, cirrhosis, phosphorus poisoning) and is associated with the excretion of sugar in the urine.

Glycosuria and hyperglycemia occur in mechanical asphyxia and in carbon monoxide poisoning, obviously as a result of increased glycogenolysis, for it has been shown that no hyperglycemia or glycosuria develops in animals in which the circulation through the liver is excluded by means of an Eck fistula. Hyperglycemia and glycosuria occur in ether and chloroform anesthesia and in morphine and strychnine poisoning.

Puncture Glycosuria. In a classical experiment, Claude Bernard discovered that puncturing the medulla of the brain in the region of the floor of the fourth ventricle resulted in glycosuria. This form of experimental glycosuria has been named *la piqûre* or puncture diabetes. It is important to bear in mind that the intensity and duration of the glycosuria produced in this way depend on the amount of glycogen present in the liver at the beginning of the experiment. If the glycogen has been removed by previous starvation, glycosuria does not occur, or is very slight.

A similar effect may be obtained by stimulating the sympathetic

[79] S. Silver and M. Reiner, "Essential Fructosuria," *Arch. Internal Med.*, **54**, 412 (1934).

[80] I. Greenwald, *J. Biol. Chem.*, **88**, 1 (1930); M. Enklewitz and M. Lasker, *Am. J. Med. Sci.*, **186**, 539 (1933).

[81] *Biochem. J.*, **26**, 957 (1932).

nerve supply to the liver (electrical stimulation or injection of adrenaline, or epinephrine). These forms of glycosuria are believed to result from increased glycogenolysis.

Possibly related to these conditions are various forms of transitory glycosuria due to nervous disturbances. It is well known that a blow on the head may lead to glycosuria (traumatic glycosuria). Fright, agitation, or struggling causes hyperglycemia and the consequent excretion of sugar in the urine (psychic or emotional glycosuria). Folin[82] examined students before and after a difficult examination and found sugar in the urine of over 15 per cent. Hyperglycemia and glycosuria are uncommon in exercise with little or no emotional stress, but common in exercise with emotional stress, as on the football field.[83] Similar observations on the relation of emotion to glycosuria were made by Cannon, but others have been unable to confirm his results. Some clinicians have attempted to establish a relationship between nerve strain and the incidence of diabetes. Although the importance of nervous factors in the etiology of diabetes has probably been exaggerated, nevertheless Macleod points out the frequent occurrence of diabetes in those predisposed to neurotic conditions, or in those whose daily habits entail much nervous strain.

Renal Glycosuria and Phlorhizin Diabetes. Glycosuria due to increased renal permeability is perhaps more frequent than was imagined before it became possible to analyze the blood for its sugar content. Errors in diagnosis were no doubt common when the clinician was forced to rely solely on urine analysis. Renal diabetes is distinguished by the low concentration of sugar in the blood. The experimental production of a somewhat similar condition was accomplished in 1886 by von Mering[84] upon injecting into animals phlorhizin, a glucoside which is found in the root bark of the cherry, apple, pear, and plum trees. Just as in renal diabetes, the sugar concentration of the blood in phlorhizin diabetes frequently falls to 0.07–0.08 per cent. The animal organism, in attempting to maintain the sugar concentration of its blood within normal levels, uses up a large proportion of its stored glycogen, and when this is depleted the proteins of the tissues are called upon to supply the needed sugar. The sugar-forming amino acids are converted into glucose, but no sooner is it formed than it is excreted by the kidneys. Thus, in severe phlorhizin diabetes, after the glycogen has been used up, 3.65 grams of glucose are excreted for every gram of nitrogen. The ratio between glucose and nitrogen excretion is called the D : N or G : N ratio and has been found to be a most valuable criterion in determining the severity of the condition both in phlorhizin diabetes and in true pancreatic diabetes. When no food is taken, a D : N ratio of 3.65 is called

[82] Folin, Denis, and Smillie, *J. Biol. Chem*, **17**, 519 (1914).
[83] Edwards, Richards, and Dill, *Am. J. Physiol.*, **98**, 352 (1931).
[84] Verhandl. V. Congr. inn. Med., 1886, p. 185.

the fatal ratio, for it represents complete failure in the utilization of glucose.

A plausible explanation of the mechanism of phlorhizin glycosuria has been lacking until recently when Lundsgaard [85] showed that in phlorhizin glycosuria the kidney contains sufficient of the poison to inhibit the action of phosphatase. He has suggested that phlorhizin glycosuria may be due to the inability of the kidney to effect the synthesis of hexosephosphate, which is assumed to be an essential stage in the reabsorption of glucose from the kidney tubules.

As an experimental method, phlorhizin diabetes has proved itself most useful. In the hands of the distinguished American physiologist Graham Lusk, his numerous students, and a number of others, this was developed into a powerful tool by means of which many difficult problems in intermediary metabolism were studied. The quantitative character of these studies enhances their value considerably. The author will again have occasion to refer to these investigations in discussing the intermediary metabolism of the proteins.[86]

It is important to bear in mind that the various forms of glycosuria described in the preceding paragraphs are usually transitory and, moreover, are not true forms of diabetes.

Pancreatic Diabetes. From a clinical standpoint this is the most common and important cause of glycosuria. Impairment in the utilization of carbohydrate is the characteristic defect in pancreatic diabetes. The tendency to hyperglycemia is very pronounced and is accompanied, when the renal threshold is exceeded, by the excretion of large quantities of glucose. Carbohydrate metabolism has been considered in relation to diabetes in other connections and will be referred to again, especially in the discussion of insulin, the hormone of the pancreas (p. 465).

[85] *Biochem. Z.*, **264**, 209 (1933).

[86] An excellent review of the subject of phlorhizin diabetes has been written by T. P. Nash, *Physiol. Rev.*, **7**, 385 (1927).

CHAPTER XII

INTERMEDIARY FAT METABOLISM

The changes which fats undergo in the intestinal mucosa constitute the starting point of our discussion of intermediary fat metabolism. We have seen that during digestion fat is hydrolyzed to fatty acid and glycerol. Early in absorption the epithelial cells at the tips of the villi contain demonstrable amounts of free fatty acid, but within several hours this is replaced by neutral fat, which in the shape of fine droplets fills the cells. According to Jeker [1] this is the histological picture of the intestinal mucosa in the normal rat, but in one poisoned with phlorhizin, or iodoacetic acid, the cells at the end of six hours still contain much free fatty acid, but considerably less fat than normally. These toxic agents are believed to prevent the formation of phospholipid, a reaction which is regarded as an essential step in the resynthesis of neutral fat.[2]

As has been stated elsewhere, the greater part of the fat absorbed from the intestine is taken up by the lymphatics and enters the blood by way of the thoracic duct. That a fraction may pass into the blood directly is indicated by the occurrence of a higher concentration in the blood of the portal vein than in that of the general circulation, a difference which tends to disappear in the post-absorptive state.[3]

During short periods of starvation, the mobilization of fat from the region drained by the portal vein also raises the fat content of the portal blood, which in passing through the liver discharges some of the fat, as demonstrated by the results of analyses of the blood taken simultaneously from the portal and hepatic veins (Himwich).[4] Moreover, it is probable that the liver may also remove fat reaching it by way of the hepatic artery. Under certain conditions, notably in depancreatized, fasting animals, the situation may be reversed; i.e., the fat content of the blood of the hepatic vein may rise above that in the portal vein, indicating the withdrawal of fat from the liver.

The "lipemia," or increase in blood lipids which results from the entrance of fat into the blood, may persist for several hours. In individuals at rest the administration of 1 gram of fat per kilogram of body

[1] *Arch. ges. Physiol.*, **237**, 1 (1936); F. Verzár and L. Jeker, *ibid.*, p. 14.

[2] R. G. Sinclair, *J. Biol. Chem.*, **82**, 117 (1929); **115**, 211 (1936); C. Artom and G. Peretti, *Arch. intern. physiol.*, **42**, 61 (1935).

[3] O. Cantoni, *Boll. soc. ital. biol. sper.*, **3**, 1278 (1928).

[4] H. E. Himwich, W. H. Chambers, A. L. Hunter, and M. A. Spiers, *Am. J. Physiol.*, **99**, 619 (1931–32).

weight is followed by a slow rise in the blood-fat concentration, reaching a maximum value in 4 hours, then rapidly diminishing, with a return to the normal level in 6–7 hours. A somewhat different lipemia curve is obtained in individuals not at rest. The rise occurs more rapidly, attaining a maximum value in about 3 hours, which is somewhat below the maximum obtained in the resting individual.[5]

During fat absorption there is a definite increase in the concentration of phospholipids (lecithin, sphingomyelin, etc.) and cholesterol esters. In human subjects, Man and Gildea [6] observed that, within 4 to 6 hours after the ingestion of 3.54 grams of fat per kilogram of body weight, the fatty acids in the serum increased to 30 to 90 per cent above the initial level. The average increase in phospholipid fatty acids was 18 per cent. The ingestion of a balanced meal, which included fat, resulted in an average rise of 21 per cent in total fatty acids, of which from 5 to 24 per cent was combined as phospholipid.[7]

A special significance has been attached to the cholesterol esters and the phospholipids of the blood. It has been assumed that, in these combinations, the transport and interchange of the fatty acids between the tissues are facilitated. It has also been suggested that the formation of these compounds represents an early stage in the intermediary metabolism of the fatty acids. This question will be considered in more detail shortly.

Following a fat-containing meal, there is a marked increase in the number of the so-called "chylomicrons," [8] which is more or less proportional to the increase in fat content. The chylomicrons are very small fat droplets, 1 micron, or less, in diameter, and are invisible to the naked eye. Not infrequently, during absorption, as well as in certain pathological conditions (anemia, nephrosis, etc.), the plasma assumes a milky appearance due to the presence of much larger fat globules than the chylomicrons. Generally this is associated with an unusually high

[5] N. I. Nissen, *Acta Med. Scand.*, **74**, 566 (1931); cited by W. R. Bloor, *Ann. Rev. Biochem*, **2**, 149 (1933).

[6] *J. Biol. Chem.*, **99**, 61 (1932–33); see also I. L. Chaikoff, *et al.*, *J. Clin. Investigation*, **13**, 1 (1934).

[7] Channon and Collinson have analyzed beef blood obtained at a fasting level. The principal components were phosphatide, cholesterol, cholesterol esters, and an unsaponifiable fraction. Only a small fraction of the total was found to be in combination as neutral fat (*Biochem. J.*, **23**, 666, 1212 [1929]). Compare with Bloor's analyses (*J. Biol. Chem.*, **59**, 543 [1924]).

The data of Man and Gildea[6] are also of interest in this connection. Human serum obtained before breakfast shows the following distribution of fatty acids:

Subject No.	I	II	III	IV	V	VI	VII	VIII	IX
Phospholipid fatty acids, m.eq	4.6	6.5	6.5	5.6	5.2	5.8	5.0	5.1	5.8
Non-phospholipid fatty acids, m.eq	5.8	8.3	9.4	7.8	5.0	7.6	5.2	7.2	7.2

Assuming the cholesterol content to be 160–200 mg. per 100 cc., or approximately 4–5 m.eq., and that of this 2–4 m.eq. is present as esters, it follows that the fatty acids in serum present in combination as fat constitute about 30 per cent of the total.

[8] S. H. Gage and P. A. Fish, *Am. J. Anat.*, **34**, 1 (1924).

lipid content, but the correlation is inconstant, and it is therefore probable that other factors are involved which affect the degree of dispersion of the lipids.

Storage of Fat. The storage of fat may occur in many regions of the body, but especially in the superficial fascia under the skin where it is present as a layer an inch or more in thickness. This layer of fat is called the *panniculus adiposus.* Large amounts of fat occur, likewise, in the intermuscular connective tissue, omentum and mesentery, and in association with the internal organs, such as the lungs, heart, kidneys, ovaries, testes, and liver. The fat in the adipose tissue of any given species is normally characteristic of that species, but the deposition of fat foreign to an animal may occur under certain conditions. A classical experiment showing this is that of Lebedeff,[9] who starved two dogs until their reserve fat was nearly used up. One dog was then fed mutton tallow and the other linseed oil, with the result that the fat deposited in the adipose tissue of the first dog resembled mutton fat, whereas the fat laid down in the second animal was liquid at 0° C. and contained larger amounts of unsaturated fatty acids than is normal for dog fat. In a similar experiment, Munk [10] fed a previously starved dog rape-seed oil and was able to demonstrate the deposition of the triglyceride of erucic acid ($C_{22}H_{42}O_2$). Eckstein [11] has also shown that the nature of the fat deposited is influenced by the fats of the diet, and he has been able to demonstrate the deposition of the myristyl radical both in the hides and carcasses of rats fed with myristic acid. However, the fat deposited on diets containing tributyrin or tricaproin did not contain the butyryl or caproyl radical, although it differed from the fat synthesized from fat-free precursors, namely protein and carbohydrate. The difference was in the degree of unsaturation rather than in the saponification numbers, as might be supposed. On a practically fat-free diet Eckstein's rats deposited fat having on an average an iodine number of 68 and a saponification number of 194, whereas on the tricaproin diet these values were 59 and 191, respectively. Brominized and iodized fats, obtained by treating fats containing unsaturated fatty acids with iodine or bromine, have been fed previously starved animals and later recovered in their bodies.

Schoenheimer and Rittenberg [12] fed mice a diet comprising various amounts of deuterium-containing fats. Deuterium is the heavy isotope of hydrogen and can be made to replace ordinary hydrogen in compounds. Physiologically important substances have been prepared containing the heavier isotope. For example, stearic acid 6–7–9–10d_4 has been prepared from linoleic acid. As the concentration of deuterium may be determined accurately in tissues, as well as in substances isolated

[9] *Arch. ges. Physiol.*, **31**, 11 (1883).
[10] *Arch. path. Anat. Physiol.*, **95**, 407 (1884).
[11] *J. Biol. Chem.*, **81**, 613 (1929); **84**, 353 (1929).
[12] *Ibid.*, **111**, 163, 175 (1935).

from tissues, it becomes possible to trace the fate of certain substances in metabolism. In this manner, Schoenheimer and Rittenberg demonstrated that the largest part of the diet fat, even when it is present in small quantities, is deposited in the fat depots before it is utilized.

In a series of experiments similar to those of Eckstein, Powell [13] fed tricaprylin to rats. Only traces of caprylic acid were deposited, but, as in the case of feeding butyric and caproic acids, there was a distinct lowering of the iodine number and very little change in the saponification number. However, when tricaprin was fed, capric acid comprised 15 per cent of the fatty acids of the depot fat, which was characterized by a high saponification number (216 as compared with 198 in the controls) and an even lower iodine number (47.7 as compared with 63.3 in the controls). Similar results were obtained with trilaurin; lauric acid comprised as much as 25 per cent of the depot fat. The iodine number was 44.8 and the saponification number 218.

These facts have an important bearing in relation to stock-raising. In certain parts of this country, the diet of hogs is composed largely of meal prepared from cottonseed or peanuts. This food may so modify the consistency of the fat laid down by these animals as to affect the marketability of the lard and other products. Ellis and co-workers [14] have shown that corn, peanut, and soybean oils, when fed to hogs to the extent of 4 per cent of the ration, have a distinct softening effect on the body fat, whereas a similar amount of cottonseed oil produces a hard fat. Increasing the cottonseed oil to 8 and 12 per cent levels results in the deposition of a softer, more unsaturated fat, containing a much greater proportion of linoleic acid. The rate of fat storage in hogs is not altered even on rations practically devoid of fat, but the fat deposited under these conditions is hard and the linoleic acid content is conspicuously low.

[13] *Ibid.*, **89**, 547 (1930); **95**, 43 (1932).

[14] *J. Biol. Chem.*, **69**, 219, 239 (1926); see also N. R. Ellis and O. G. Hankins, *ibid.*, **66**, 101 (1925); Ellis and J. H. Zeller, *ibid.*, **89**, 185 (1930); Ellis, C. S. Rothwell, and W. O. Pool, *ibid.*, **92**, 385 (1931); J. M. Spadola and N. R. Ellis, *ibid.*, **113**, 205 (1936).

One hog kept on a basal corn diet and a 4 per cent level of cottonseed oil deposited a back fat having the following characteristics: iodine number, 64; melting-point, 46.2 °C.; saponification number, 196.3. The fatty acid distribution in per cent was: oleic acid, 34.5; linoleic, 16.4; total saturated, 44.5. In contrast are the following data: for the fat deposited on the 12 per cent level; iodine number, 83.8; melting-point, 35.5° C; saponification number, 195.6. Fatty acids: oleic, 32.3; linoleic, 28.2; total saturated, 34.9.

One hog kept on a low fat ration for 257 days attained a weight of 282 lb. The body fat had an iodine number of 55.1, a melting-point of 37.6° C., and a saponification number of 195. The distribution of the fatty acids was as follows: oleic, 58.9; linoleic, 1.3; arachidonic, 0.02; palmitic, 24.3; stearic, 10 3; myristic, 0.7 per cent.

Bhattacharya and Hilditch have pointed out that, in the deposition of hog fat under various circumstances, there is a marked tendency to approximate constancy in the molar content of the C_{18} acids in spite of variation in the total proportion of saturated to unsaturated acids (*Biochem. J.*, **25**, 1954 [1931]).

This problem has also been studied in rats by Anderson and Mendel.[15] In their experiments, the diet was very carefully controlled and only the fat, or other nutrients available for fat formation, were varied. They found that when soybean oil, cottonseed oil, or peanut oil was fed, the resulting body fat resembled the food fat. When butter fat or coconut oil were fed, the deposited fat differed from the food fat, having a lower iodine number. Anderson and Mendel point out that, even after a particular type of "soft" body fat has been developed, it is possible to alter its chemical make-up by changing to a "hardening" diet rich in carbohydrate. This process is apparently due first to the gradual depletion of the soft fat and the subsequent deposition in its place of the harder variety. This process is relatively slow, but it may be materially hastened if prior to changing the diet the fat reserves are partly used up by a short period of fasting.

Nearly forty years ago, Henriques and Hansen[16] made similar, though not so extensive, observations on hogs. In one of their experiments, they fed a hog on barley and another on maize. The fat laid down in the connective tissue of the former had an iodine number of 57.7 and a melting-point of 27.4°, whereas the fat of the corn-fed hog had an iodine value of 75.6 and a melting-point of 23°. Despite the profound effect which diet has on the nature of the fat deposited, it is nevertheless true that the fat in any given species is fairly constant in composition. This is due in large part, no doubt, to the similarity in the type of food consumed by animals of the same species.

The composition of adipose-tissue fat varies in different parts of the body. In their experiments on hogs, Henriques and Hansen showed that subcutaneous fat has a higher iodine number and a lower melting-point than perirenal fat, which in turn has a higher iodine number and a lower melting-point than omental fat. These differences may be due to temperature, for these workers have shown that the temperature of subcutaneous tissue in the hog, 1 cm. from the surface, is 33.7°; at 2 cm., it is 34.8°; and at 4 cm., 39° C. The composition of fat has been modified by altering the temperature of the environment. In one experiment, Henriques and Hansen kept three pigs from the same litter at different temperatures; the first was kept at 30–35° C. and the second at 0°; the third was also kept at 0° but was covered and kept warm with a sheepskin coat. After two months, the pigs were killed and the fat analyzed. It was found that the fat of the pig kept at 0° C. without any cover showed the highest iodine number (72.3). The pig kept at 30–35° had deposited fat having an iodine number of 69.4, whereas the clothed pig, kept at 0° C., had fat which showed the lowest iodine number of the series (67.0). There is, as yet, no satisfactory

[15] *J. Biol. Chem.*, **76**, 729 (1928); see also W. E. Anderson and H. H. Williams, "The Rôle of Fat in the Diet," *Physiol. Rev.*, **17**, 335 (1937).

[16] *Skand. Arch. Physiol.*, **11**, 151 (1901); also described in the monograph by Leathes and Raper, p. 100.

explanation which accounts for these variations. One can readily see, however, the advantage of having a relatively solid fat, of high melting-point, in the region of the back of some animals, which is so often exposed to the relatively high temperatures of the sun's rays.

Distribution of Adipose Tissue. A detailed study of the distribution and character of the fat deposited in the organism has been reported from Mendel's laboratory.[17] The following factors have been considered: diet, weight, sex, undernutrition, fasting, exercise, ovariectomy, the administration of thyroxine. The normal distribution of fat in the various depots in the female rat was approximately as follows (in per cent): subcutaneous 50, genital 20, perirenal 12, mesenteric 10, intermuscular 5, omental 3. This distribution was independent of the type of diet fed; however, the greatest amount of fat was deposited on a diet rich in fat. The degree of saturation (iodine number) of the stored fat was independent of the distribution, but was markedly influenced by the type of fat in the diet.[18] In young rats, the proportion of subcutaneous fat was larger than in the older animals. With increase in weight, the rate of storage was found to be increased. The female rats stored more fat in the genital depots, whereas male rats accumulated a greater proportion of fat in the perirenal fat depots. Undernutrition or forced activity in animals kept on a starch diet resulted in a decreased proportion of the genital fat. Intermuscular fat increased in proportion as a result of physical activity, but even on an *ad libitum* diet, the exercised animals became thin. Neither weight, sex, undernutrition, fasting, nor exercise influenced the iodine number of the fat deposited. As compared with non-spayed controls, ovariectomized rats stored less fat in the genital depots, but more in the subcutaneous tissues. Daily doses of thyroxine markedly diminished the total fat deposited. The distribution was approximately the same as in the controls, but the fat deposited was considerably more unsaturated.

It is probable that not all the fat in the body has the same physiological significance. A distinction has been made by a number of

[17] L. L. Reed, F. Yamaguchi, W. E. Anderson, and L. B. Mendel, *J. Biol. Chem.*, **87**, 147 (1930); Reed, Anderson, and Mendel, *ibid.*, **96**, 313 (1932).

[18] Thus, in rats kept inactive and given an unlimited amount of food, the following differences in the iodine number were observed (average values for 6 animals in each group):

	Intermuscular	Genital	Subcutaneous	Perirenal	Mesenteric	Omental
I. Starch diet.....	57.1	55.4	56.6	53.8	50.2	
II. Soybean oil.....	105.1	114.0	111.3	118.3	110.0	100.9
III. Coconut oil.....	29.7	28.2	29.4	28.6	25.8	26.2

workers (Mayer, Schaeffer,[19] Terroine,[20] and others) between the so-called *élément constant* and *élément variable.* The tissues of animals that have starved to death still contain a certain amount of fat, which seems to be fairly constant for any given species. In the mouse, for example, about 23 per cent of the dry weight of the animal consists of fatty acids, whereas in the chicken the fatty acids constitute about 25 per cent of the dry weight. It is believed that a certain amount of fat (and especially of the phospholipid fraction) is an essential component of protoplasm, and that this cannot be reduced without causing death. This is the *élément constant.* On the other hand, the reserve fat is variable in amount, depending on the state of nutrition and other factors. The fatty acids in reserve or storage fat are present in combination with glycerol as neutral fat. Such fat, because it varies in amount, has been called the *élément variable* by the group of workers mentioned above.[21]

Abnormal Fat Deposit in Liver. Blatherwick[22] has made the interesting observation that rats fed diets containing whole liver develop livers containing large amounts of fat and cholesterol esters. The effect seems to be related to the cholesterol contained in liver, and indeed it was found possible to reproduce the condition of "fatty liver" by feeding cholesterol. This has been confirmed by Chanutin and Ludewig,[23] who have shown, moreover, that other tissues (kidney, heart, brain, etc.) are practically unaffected.

Relation of Lecithin and Choline to Fat Deposition in the Liver. Depancreatized dogs receiving insulin, but kept on a diet of cane sugar and lean beef develop, after a variable period, failure of liver function, characterized by fatty infiltration and degeneration. The alleviation of this critical condition may be brought about by the addition of lecithin to the diet. Stimulated by this interesting observation, Best and associates demonstrated that normal rats when fed a diet rich in fat and low in protein showed an abnormal accumulation of fat in the liver. This was not observed in control animals receiving a similar diet together with lecithin. A difference in the composition of the fat deposited was indicated by the iodine number (average of 100 in the experimental group, as contrasted with an average of approximately 132 in the control group). Further investigation revealed that the effect of lecithin was due to the choline component of the molecule. Betaine was found to exert a similar effect, while amino ethyl alcohol, the nitrogenous base of cephalin, was without action. Choline diminishes but does not

[19] *J. physiol. path. gén.*, **15**, 510, 535, 773, 984 (1913); **16**, 1, 23 (1914).

[20] E. F. Terroine, *J. physiol. path. gén.*, **16**, 384, 212 (1914); "Physiologie des substances grasses," Paris (1919). A recent discussion of the subject by Terroine is to be found in *Ann. Rev. Biochem.*, **5**, 227 (1936).

[21] Monaghan (*J. Biol. Chem.*, **98**, 21 [1932]) reduced the phospholipid content of the tissues in fasting rats to a value 60 per cent below normal and accordingly has raised a question as to the validity of the conception of the *élément constant.*

[22] *J. Biol. Chem.*, **97**, Proc. xxxiii (1932); **100**, xviii (1933); **103**, 93 (1933).

[23] *Ibid.*, **102**, 57 (1933).

prevent the abnormal deposition of fat in the liver (rat), produced by feeding cholesterol.[24]

It is of interest that increasing the protein of the diet diminishes the deposition of fat, but accentuates the deposition of cholesterol esters in the liver.

Human Fat. Cathcart and Cuthbertson [25] have analyzed the abdominal fat of a normal subject and obtained an average iodine value of 68.4. Similar analyses of fat from two stout women yielded an average iodine value of 71. The liver fat was freed from other lipids. In the normal individual, the iodine number was 127. Marked fatty change was visible in the livers of the obese women, but the iodine number of the fat was only 73. The average iodine number of normal muscle fat was 74.

Obesity. Obesity is usually the result of overnutrition, lack of exercise, or both. It is a matter of common observation, however, that certain individuals increase in weight despite an *apparently* moderate diet, while others remain thin in spite of all efforts to gain weight by overeating. Then there is the average individual who seems to make no conscious attempt to control his diet but whose weight remains fairly constant over a period of many years.

Grafe [26] advanced the view that in normal individuals the intake of excessive amounts of food results in an increased metabolism, so that the extra energy is not stored and the weight is therefore maintained relatively constant. Accordingly he attributed obesity to the failure of this alleged metabolism-stimulating mechanism, whereas leanness he considered to be due to an over-response to a normal stimulus. A distinction was drawn by Grafe between so-called *exogenous obesity* and the *endogenous* or *constitutional* type, the former being due presumably to inactivity and overnutrition, whereas the latter he believed to be in some way associated with intrinsic factors such as endocrine derangement. Disease of the hypophysis, castration, the menopause, myxe-

[24] J. M. Hershey, and S. Soskin, *Am. J. Physiol.*, **98**, 74 (1931); C. H. Best and Hershey, *J. Physiol.*, **75**, 49 (1932); Best, Hershey, and M. E. Huntsman, *ibid.*, **75**, 56 (1932); Best and Huntsman, *ibid.*, **75**, 405 (1932); Best and Ridout, *ibid.*, **78**, 415 (1933); Best, G. C. Ferguson, and Hershey, *ibid.*, **79**, 94 (1933); Best, M. E. Mawson, and Ridout, *ibid.*, **86**, 315 (1936); A. W. Beeston, H. J. Channon, and H. Wilkinson, *Biochem. J.*, **29**, 2659 (1935).

Dragstedt, van Prohaska and Harms, *Am. J. Physiol.*, **117**, 175 (1936), prepared an alcoholic extract of beef pancreas, which, when administered orally to depancreatized dogs treated with insulin, permitted survival and prevented or relieved the fatty degeneration and infiltration of the livers of these animals. Dragstedt attributes this effect on fat transport and utilization to a specific hormone for which he has suggested the name " lipocaic."

[25] *J. Physiol.*, **72**, 349 (1931); see also A. J. McAmis and W. E. Anderson, *Proc. Soc. Exptl. Biol. Med.*, **28**, 749 (1930–31), and H. C. Eckstein, *J. Biol. Chem.*, **64**, 797 (1925).

[26] E. Grafe and D. Graham, *Z. physiol. Chem.*, **73**, 1 (1911); Grafe, *Ergebnisse Physiol.*, **21**, part 2, 197, 282 (1923).

dema, and other physiological and pathological disturbances are usually, but not invariably, accompanied by the deposition of an abnormal amount of fat.

Grafe's so-called *Luxuskonsumption* hypothesis has been challenged by Wiley and Newburgh.[27] They overfed an unusually thin subject, but observed no increase in metabolism above that due to the specific dynamic action of the extra food (p. 530). Moreover, the subject gained weight rapidly. Newburgh is of the opinion that obesity is never directly caused by an abnormal metabolism, but that it is always due to food habits not adjusted to the metabolic requirement. Either more food is eaten than is normally needed, or the intake is not sufficiently reduced in response to a lowered requirement, from whatever cause.

Diabetes occurs not infrequently in obese individuals past middle age, and it has often been suggested that obesity may be regarded in many cases as a precursor and possibly even a cause of diabetes.[28]

The hereditary factor in obesity has been recently stressed by Gurney.[29]

Relation of Phospholipids to Fat Metabolism. Lowe [30] was apparently the first to suggest that lecithin served as a conveying mechanism in the interchange of fat in the body, a view which received much support from the later observation of Bloor [31] that the phospholipid concentration increased markedly during alimentary lipemia. With the demonstration that cholesterol esters likewise increased during fat absorption, the formation of these esters has also been accepted as a possible mechanism in the transportation of fatty acids.

It has been shown repeatedly, in feeding experiments with various fats, that as a result of fat absorption the fatty acids of the liver always have a higher iodine number than the fatty acids of the ingested fat. Particularly does this affect the phospholipid fraction, which though usually unchanged in percentage is altered chemically by becoming more unsaturated. This occurs even on feeding so unsaturated a fat as cod-liver oil. After absorption, the iodine number of the liver phospholipid is higher than that of the cod-liver oil and much higher than that of the liver phospholipid, in the fasting state.

Such facts have led to the view, first advanced by Leathes,[32] that

[27] *J. Clin. Investigation*, **10**, 733 (1931); L. H. Newburgh and M. W. Johnston, *ibid.*, **8**, 197 (1929–30).

[28] The student is referred to the editorial entitled "Obesity and Diabetes," *J. Am. Med. Assoc.*, **95**, 202 (1930); see also S. F. Adams, *J. Nutrition*, **1**, 339 (1929); E. P. Joslin, *New England J. Med.*, **209**, 519 (1933).

[29] *Arch. Internal Med.*, **57**, 557 (1936).

[30] *Biol. Zentr.*, **11**, 269 (1891).

[31] W. R. Bloor, *J. Biol. Chem.*, **24**, 447 (1916).

[32] J. B. Leathes and L. Meyer-Wedell, *J. Physiol.*, **38**, xxxviii (1909); E. L. Kennaway and Leathes, *Proc. Roy. Soc. Med., Pathol. Sect.*, **2**, 136 (1909); Leathes and H. S. Raper, "The Fats," London, 1925.

the liver participates in fat metabolism, its function being that of converting the fatty acids (in the form of phospholipids, etc.) which it absorbs into more highly unsaturated fatty acids, by a process of dehydrogenation. It may be supposed that in this way the fats are rendered more useful to other tissues because the ease with which fatty acid chains are broken up and oxidized is presumably determined by the number of double linkages.

This conception of the rôle of phospholipids in fat metabolism has been accepted by many biochemists, but has recently been subjected to criticism by Sinclair,[33] who found that, if rats previously kept on a fat-poor diet are fed cod-liver oil, the degree of unsaturation of the constituent fatty acids of the phospholipids in the liver and muscle is markedly increased. The change is rapid, particularly in the liver, where the replacement of the more saturated by the more unsaturated fatty acids is essentially complete within three days. The rate of turnover in the rest of the carcass is slower, only about 50 per cent replacement occurring in the same period. Moreover, the rate of turnover is not increased by reducing the temperature of the environment, as might be expected from the fact that metabolism is intensified at low temperatures. In contrast to the rapid rate with which the degree of unsaturation was increased from a lower to a higher level, the reverse process, namely the decrease from a high level of unsaturation to a low level, was found to be very slow.[34] This was attempted by fasting or by changing to a fat-free diet or to one containing coconut oil.

It is argued that, if phospholipid were involved in intermediary fat metabolism, the rate of turnover would be rapid; that it is slow signifies a relation, not to the principal path of fatty acid oxidation, but to the " wear and tear " of the tissues only. Excepting the liver and blood, the phospholipid content of organs and tissues remains relatively constant under a large variety of conditions. Each organ is characterized by a certain amount of phospholipid, which according to Sinclair is uninfluenced by the intensity of fat metabolism in that organ. This lack of dependence should not be confused with the relation that exists between phospholipid content and functional activity of tissues (p. 355). It should also be made clear that Sinclair's conclusion, while denying the rôle of phospholipids in intermediary fat metabolism, does not minimize

[33] *J. Biol. Chem.*, **95**, 393 (1932); **100**, lxxxvii (1933); **115**, 211 (1936); *Physiol. Rev.*, **14**, 351 (1934).

[34] This effect seems to be independent of the amount of cod-liver oil fed. Indeed, if either a fat-free or fat-poor diet is supplemented by one drop of cod-liver oil per day, it results in a marked increase in the iodine number of the phospholipids, particularly in the liver, where it almost attains the maximum value. These small fat supplements do not, however, influence the degree of unsaturation of the neutral fat. Now, it has been observed that, on a fat-free diet, growth is retarded. Sinclair accordingly raises the question of a possible casual relationship between the low degree of unsaturation of the tissue phospholipids and the subnormal growth of rats on a fat-free diet (*J. Biol. Chem.*, **96**, 103 [1932]).

their importance in the transportation of fatty acids, a function that may be represented by the equation:

$$\text{Fatty acids} \rightleftharpoons \text{phospholipid} \rightleftharpoons \text{neutral fat}$$

According to Sinclair, only the plasma phospholipid serves as a mechanism for the transport of fatty acids to the tissues where they are stored or oxidized. In the red cells the increase in phospholipid is insignificant, indicating that they do not participate in fat transport.

The theory has been proposed that phospholipids are concerned in the structural make-up and activity of the cells. Significance is attached to the physicochemical properties of phospholipids: their solubility in fat, combined with miscibility in water, low surface tension, etc. It has also been stressed that, owing to the presence of unsaturated linkages in the constituent fatty acids, the phospholipids function as oxygen-transport agents by virtue of easily reversible oxidation at the double (C=C) bonds. A consideration of these theories points to the probability that the phospholipids are intimately involved in cellular activity; however, the relative importance of the various mechanisms cannot be assessed with any degree of precision on the basis of existing knowledge.

Factors Influencing Composition of Phospholipids in Tissues. Storage or depot fat, which is relatively resistant to oxidation, consists almost entirely of the triglycerides of fatty acids, only a small proportion of which are highly unsaturated. The amount of phospholipid is small and the iodine number varies between 60 and 70, being usually about 65.[35] In contrast to this is the composition of the lipids present in the liver. Bloor,[36] who has made a detailed study of the tissues of the beef, gives 87 for the average iodine number of the mixed fatty acids of the fat (acetone-soluble) fraction of the liver. The mixed fatty acids of the cephalin and lecithin fractions have higher iodine numbers, 119 and 108, respectively. Somewhat higher values are given by Theis,[37] whose results show a constant relation of phospholipid to neutral fat in the liver, as indicated by the following figures:

	Phospholipid, Per Cent	Neutral Fat, Per Cent
Beef liver	55	45
Rabbit liver	55–65	35–45
Human liver	60	40

[35] H. C. Eckstein, *J. Biol. Chem.*, **64**, 797 (1925).

[36] *Ibid.*, **56**, 711 (1923); **59**, 543 (1924); **68**, 33 (1926); **72**, 327 (1927); **80**, 443 (1928).

[37] *Ibid.*, **76**, 107 (1928); **77**, 75 (1928); **82**, 327 (1929).

However, if the liver is damaged or diseased, this relation is altered; the proportion of phospholipid is greatly diminished apparently because of failure to convert neutral fat to phospholipid. In liver injury the desaturation of the fatty acids is not carried as far as normally, for according to the data given by Theis the iodine number of the various lipid fractions is markedly diminished.

The difference in the degree of unsaturation of the liver and depot lipids is not due solely to the predominance of the phospholipid fraction in the former. Bloor and Snider [38] have shown that the neutral fat of the liver is itself relatively unsaturated, having a higher iodine number than the fat of the depots, or of any other organ examined (heart, kidney, lung). Although this finding is in harmony with Leathes' conception of the liver as a desaturating organ, Bloor [39] has suggested the possibility that the unsaturation of the liver lipids may be due to the removal, by the liver, from the blood which reaches it, of fatty acids of high iodine number.[40]

Diet is an important factor influencing the composition of phospholipids in animal tissues. Thus, Sinclair,[41] in feeding experiments on cats, compared the effect of an exclusive diet of either beef kidney or beef muscle.[42] As regards the phospholipid content in the various tissues no difference was observed except in the liver, more phospholipid being present in the livers of the animals on the kidney diet than in the livers of those maintained on the muscle diet. However, with the exception of the brain there was a marked difference in the degree of unsaturation of the phospholipids of the various tissues in the two groups, as shown by the following data[43] for the iodine number:

	Beef Kidney	Beef Muscle
Brain	105 ± 2 4	100 ± 1.8
Liver	145 ± 2 5	124 ± 8
Kidney	112 ± 1 9	95 ± 3 8
Heart	140 ± 3 6	121 ± 3 2.
Striated muscle	131 ± 6 3	108 ± 2 7

[38] *Ibid.*, **87**, 399 (1930).

[39] *Ann. Rev. Biochem.*, **1**, 267 (1932).

[40] A similar objection to Leathes' hypothesis was voiced by some of the earlier students of the subject. To answer it, Raper fed cats coconut oil, a very saturated fat (iodine number of about 8) containing a high percentage of volatile fatty acids. Raper recovered from the liver volatile fatty acids which were more unsaturated than those contained in the coconut oil.

[41] *J. Biol. Chem.*, **86**, 579 (1930).

[42] The iodine number of beef kidney phospholipid is about 98 and of beef muscle phospholipid 82. Beef kidney contains about 7 times as much phospholipid as beef muscle.

[43] Compare with E. F. Terroine, and P. Belin, *Bull. soc. chim. biol.*, **9**, 12 (1927); Terroine and C. Hatterer, *ibid.*, **12**, 674 (1930).

In a comparison of the composition (iodine number) of the phospholipids and neutral fats in the tissues of rats maintained on various diets, Sinclair [44] found the most saturated phospholipids (iodine number of about 100) in rats kept on a fat-free or fat-poor diet. The most unsaturated phospholipids were observed in animals receiving cod-liver oil. But it seemed that any fat, including coconut oil, produced more unsaturated phospholipids than those synthesized on the "fat-free" diet. There was a rough parallelism between the iodine number of the neutral fat stored by the animal and that of the food fat, but no such relation was demonstrable for the tissue phospholipids.

Growth is another important factor, according to Sinclair.[45] It appears from his studies on rats that the phospholipid content, in relation to the tissue solids, decreases rapidly after birth, the period of most rapid decline coinciding with the period of most rapid growth.[46]

Activity. In comparing various muscles of the beef, Bloor found that the more active the muscle, the higher its percentage content of phospholipid and unsaponifiable substance. A similar relation can be made out for different organs (kidney, pancreas, lung, brain), for, according to Bloor, the arrangement of the organs in the order of their phospholipid content gives a series which represents also the order of their functional activity. Heart muscle of the wild rabbit contains two and one-half times as much phospholipid as the gastrocnemius muscle.

In the corpus luteum of the pig the phospholipid content was about three times as great during its active state in ovulation and pregnancy as in the resting state. Mammary glands (rabbit) at the end of pregnancy had twice the phospholipid content of the resting glands. Malignant tumors had about three times the content of phospholipid and twice the amount of cholesterol possessed by benign tumors.[47]

Oxidation of Fatty Acids. *β-Oxidation.* Normally, the fatty acids are completely oxidized to carbon dioxide and water. Of the intermediate steps in the process, little was known until Knoop [48] published the results of his investigations in 1904. Earlier attempts had been made to trace the fate of fatty acids in metabolism, by feeding the lower members to animals and then examining the urine for end-products. These experiments were unsuccessful because the fatty acids were either completely oxidized or partly excreted unchanged. Knoop, however, conceived the idea of feeding phenyl derivatives of the lower fatty acids.

[44] *J. Biol. Chem.*, **92**, 245 (1931).

[45] *Ibid.*, **88**, 575 (1930).

[46] On the contrary, Monaghan (*ibid.*, **98**, 21 [1932]) found that there is a constant value for the phospholipid fatty acids in a given tissue of rats which are growing normally on an adequate diet. However, any type of dietary deficiency associated with a falling off in the rate of growth is also accompanied by a decrease of the phospholipid fatty acid content of the tissues.

[47] W. R. Bloor and R. H. Snider, *J. Biol. Chem.*, **107**, 459 (1934); Bloor, *Proc. Soc. Expll. Biol. Med.*, **27**, 294 (1929–30).

[48] *Beitr. chem. Physiol. Path.*, **6**, 150 (1904)

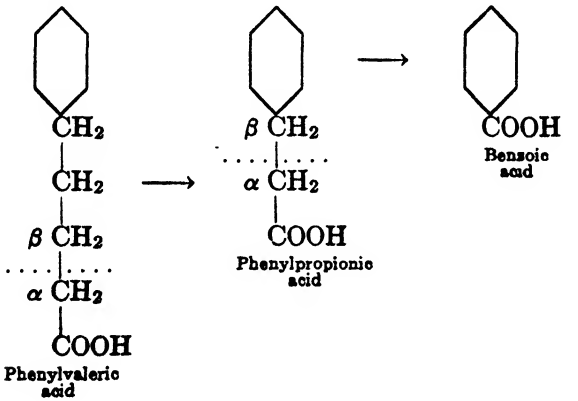
Benzoic acid is not oxidized in the body, and when fed, is excreted in the urine in combination, partly with glycine as hippuric acid.[49] The reaction is represented by the following equation:

$$C_6H_5 \cdot COOH + CH_2NH_2 \cdot COOH = C_6H_5CONHCH_2COOH + H_2O$$

Phenylacetic acid is likewise resistant to oxidation and is detoxified to form phenaceturic acid, in which form it is excreted. In man, according to Thierfelder and Sherwin,[50] phenylacetic acid is conjugated to form phenacetylglutamine. The formation of phenaceturic acid may be written:

$$C_6H_5CH_2COOH + CH_2NH_2 \cdot COOH$$
$$= C_6H_5CH_2CO \cdot NH \cdot CH_2COOH + H_2O$$

However, on feeding phenylpropionic acid, Knoop found hippuric acid in the urine, showing that two carbon atoms of the side chain had been removed by oxidation. Phenylbutyric acid yielded phenaceturic acid, and phenylvaleric acid gave rise in the body to benzoic acid, which was in turn converted into hippuric acid and excreted. Knoop therefore concluded that the oxidation of a fatty-acid chain occurs at the β-carbon atom. The oxidation of phenylvaleric acid may be represented as follows:



Other evidence is available in support of Knoop's hypothesis of β-oxidation. In diabetes, the oxidation of fat is incomplete, with the result that products of incomplete oxidation are found in the urine. These are β-hydroxybutyric acid and acetoacetic acid. In normal

[49] Benzoic acid formed from phenylpropionic acid in the dog is not excreted solely in combination with glycine. According to Quick (*J. Biol. Chem.* **77**, 581; **80**, 515, 527, 535 [1928]; **98**, 537 [1932]), a much larger proportion is conjugated with glycuronic acid, the ratio being 3 : 1.

[50] *Ber.*, **47**, 2630 (1914). According to Quick, phenylacetic acid also forms a conjugation product with glycuronic acid.

metabolism, these are further oxidized to carbon dioxide and water; but in diabetes, or more correctly, in disturbed glucose metabolism, they are partly converted into acetone, the remainder being excreted unchanged. Collectively, they are called the " acetone bodies." A better term would be " acetone substances " or " acetone compounds." Both hydroxybutyric and acetoacetic acids are excreted partly as salts. This accounts for the loss of fixed base from the body when these substances are formed in metabolism. The prevailing opinion has been that the formation of acetone bodies can be explained only by assuming that the long-chain fatty acids, of an even number of carbon atoms, are oxidized successively at the β-carbon atom.[51]

Multiple Alternate Oxidation. It has been determined that butyric acid yields less acetoacetic acid than certain higher fatty acids, such as caprylic, capric, and lauric. This result was first obtained by Jowett and Quastel[52] in experiments in which oxidation of the fatty acids was brought about by liver slices, and has been confirmed in metabolism studies on the intact animal (Deuel, *et al.*,[53] Brentano and Markees[54]). The data thus obtained may be explained on the basis that oxidation proceeds simultaneously along the whole chain of a fatty-acid molecule, at alternate carbon atoms (β, δ, ζ, etc.), with the formation ultimately of acetoacetic acid and other acid products. Jowett and Quastel's hypothesis of *multiple alternate oxidation* differs therefore from Knoop's theory of β-oxidation which postulates step-by-step oxidation at the β-carbon atom and successive cleavage of the fatty-acid molecule between the α and β carbon atoms, with the loss of a molecule of acetic acid.

Multiple alternate oxidation may be illustrated by the following scheme for *n*-octanoic acid.

$$\mathrm{CH_3\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH}$$

$$\mathrm{CH_3\cdot \overset{\searrow}{C}\cdot CH_2\cdot \overset{\searrow}{C}\cdot CH_2\cdot \overset{\searrow}{C}\cdot CH_2\cdot COOH + 6H}$$

$$\mathrm{CH_3\cdot CO\cdot CH_2\cdot CO\cdot CH_2\cdot CO\cdot CH_2\cdot COOH}$$

$$\mathrm{2CH_3\cdot CO\cdot CH_2\cdot COOH} \quad \text{or} \quad \mathrm{CH_3\cdot CO\cdot CH_2\cdot COOH + 2CH_3\cdot COOH}$$

[51] Knoop's theory of β-oxidation was supported by the work of Dakin, *J. Biol. Chem.*, **4**, 77, 227, 419 (1908); **5**, 173, 303 (1908); **6**, 203, 221 (1909); **56**, 43 (1923); Embden and Marx, *Beitr. chem. Physiol. Path.*, **11**, 318 (1908); Ringer, *J. Biol. Chem.*, **14**, 43 (1913), and others.

[52] *Biochem. J.*, **29**, 2143 (1935).

Cohen has offered the suggestion that the enzyme system involved in the oxidation of fatty acids (β-oxidase system) requires a particular chemical grouping, such as CH_3CH_2CO— or —CH_2CH_2CO— or —CH_2CH_2COOH. The carbonyl group, irrespective of its location in the molecule, serves as a polar or orienting group. Oxidation occurs β to this carbonyl group. Cohen considers his scheme in line with Jowett and Quastel's theory of fatty-acid oxidation. See *J. Biol. Chem.*, **119**, 333 (1937).

[53] *J. Biol. Chem.*, **116**, 621 (1936).

[54] *Z. ges. Exptl. Med.*, **99**, 498 (1936).

ω-Oxidation. This refers to the oxidation of the terminal methyl group of fatty acids, resulting in the production of dicarboxylic acids. This new type of oxidation was discovered by Verkade and fellow-workers[55] on feeding triundecylin (triglyceride of undecoic acid, $CH_3(CH_2)_9 \cdot COOH$) to human subjects. Considerable quantities of undecanedioic acid ($COOH \cdot (CH_2)_9 \cdot COOH$) were excreted in the urine. Formation and excretion of dicarboxylic acids occurred after feeding the triglycerides of certain other fatty acids: caprylic C_8, nonylic C_9, capric C_{10}, lauric C_{12}. Apparently the fate of these fatty acids in the body is not limited to ω-oxidation; β-oxidation accompanying it results in the formation of dicarboxylic acids of a smaller number of carbon atoms than the original fatty acid. This is shown by the fact that feeding tricaprin is followed by the excretion, in addition to sebacic acid (C_{10}), of small amounts of C_6 and C_8 dicarboxylic acids. Combined ω- and β-oxidation has been further ascertained by the administration to dogs of purified dicarboxylic acids and recovering these, as well as their lower homologues, in the urine. Thus, feeding sebacic acid was followed by the excretion of adipic (C_6) and suberic acids in addition to the substance fed. After feeding undecanoic acid (C_{11}), the urine contained pimelic (C_7) and azelaic (C_9) acids.

No dicarboxylic acids are excreted after the administration of the higher triglycerides. This may mean either that the long-chain fatty acids do not undergo ω-oxidation, or else that this type of oxidation does occur, but that β-oxidation at both ends of the molecule (or multiple alternate oxidation) reduces the fatty acid to small fragments. That succinic acid may be produced in this manner is not impossible. The excretion, by contrast, of short-chain carboxylic acids in comparatively large amounts may be due to their relative resistance to β-oxidation.

Other Aspects of Fatty-acid Oxidation. We have seen that the metabolism of fatty acids depends on β-oxidation, according to one theory, or multiple alternate oxidation, according to another theory. In either case it is probable that the first step in the breakdown of fatty acids consists in desaturation of the molecule. This theory, proposed originally by Leathes and Wedell,[56] is consistent with the increased unsaturation (iodine number) in the tissues during metabolism. More direct evidence has been submitted recently by Schoenheimer and Rittenberg.[57] Mice were fed saturated fatty acids containing deuterium (p. 345); the fatty acids of the entire animals were fractionated, all trace of the saturated fatty acids being removed from the unsaturated fatty acids. The latter were found to have a high deuterium content, which proves that desaturation had occurred, provided the assumption is correct that no exchange of deuterium had taken place in the organism.

[55] P. E. Verkade and J. van der Lee, *Biochem. J.*, **28**, 31 (1934); *Z. physiol. Chem.*, **237**, 186 (1935); *Bull. soc. chim. biol.*, **18**, 989 (1936).

[56] *J. Physiol.*, **38**, xxxviii (1909).

[57] *J. Biol. Chem.*, **113**, 505 (1936).

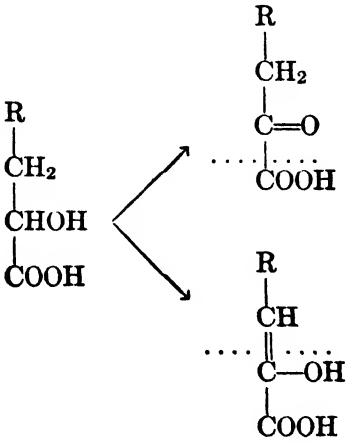
The shifting of double bonds is not an unfamiliar phenomenon. Quick [58] has shown that phenylisocrotonic acid, $C_6H_5 \cdot CH{=}CH \cdot CH_2 \cdot COOH$, is handled by the organism exactly like phenylbutyric acid. Although the double bond is between the β- and γ-carbon atoms, the split occurs between the α- and β-positions. This observation is significant in showing that the oxidation does not necessarily occur at the point of unsaturation.

Witzemann [59] has investigated the oxidation by potassium permanganate of the sodium salts of a series of α-hydroxy-straight-chain-fatty acids. It has been known for a long time that α-hydroxybutyric acid is partly oxidized with the loss of 1 carbon atom and partly with the loss of 2 carbon atoms. This has been confirmed by Witzemann, who found, moreover, that the two types of breakdown occur almost equally. These may be summarized as follows:

A. $CH_3CH_2CHOHCOOH + O_2 \rightarrow CH_3CH_2COOH + CO_2 + H_2O$

B. $CH_3CH_2CHOHCOOH + 5O \rightarrow CH_3COOH + 2CO_2 + 2H_2O$

It is pointed out that α-hydroxybutyric acid occupies a pivotal position between lactic acid (α-hydroxypropionic acid) which is oxidized exclusively by the loss of 1 carbon atom, and the higher α-hydroxy homologues, in which the loss of 2 carbon atoms at a time occurs readily. Particularly remarkable is the fact that, in the presence of an excess of alkali (1 molecule of alkali per molecule of the sodium salt of the fatty acid), the point of rupture of the fatty-acid chain shifts from a loss of 1 carbon atom largely to a loss of 2 carbon atoms at a time. This result was obtained even with lactic acid. According to Witzemann, the loss of 1 carbon atom is due to the oxidation of the α-keto acid, while the loss of 2 carbons follows the oxidation of its enol isomer. This may be represented by the following formulas:

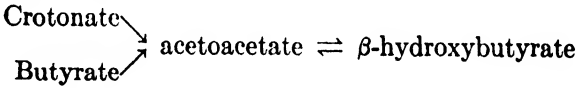


[58] *J. Biol. Chem.*, **77**, 581 (1928). [59] *Ibid.*, **95**, 219, 247 (1932).

In short, the work of Witzemann indicates that the exclusion of α-oxidation in fat metabolism is not necessarily justified, inasmuch as α-hydroxy fatty acids may lose more than one carbon atom on oxidation.

When either butyric or β-hydroxybutyric acid is administered to a diabetic animal, acetoacetic acid is formed. In the same manner, the conversion of acetoacetic acid into β-hydroxybutyric acid may be demonstrated; indeed, the rate of this reaction is more rapid than the reverse transformation of β-hydroxybutyric to acetoacetic acid. The formation of both of these products has aroused considerable interest in the question of whether the keto or the hydroxy acid is formed first. Numerous studies of the rate of metabolism of the two acetone bodies have not definitely settled the question, but much of the evidence points to crotonic acid ($CH_3 \cdot CH{=}CH \cdot COOH$), or acetoacetic acid, rather than β-hydroxybutyric acid as the primary product. It was Dakin's view, however, that the immediate oxidation product of butyric acid may be any one of the three: β-hydroxybutyric or acetoacetic or crotonic acid.[60]

A somewhat different conclusion has been reached by Jowett and Quastel,[61] who have investigated the oxidation of these intermediates *in vitro*, using liver slices (guinea pig). These authors believe that acetoacetic acid is derived either from butyric acid, or from crotonic acid, the latter being derived from the former by dehydrogenation. The relation of β-hydroxybutyric acid to the other intermediates is as represented in the following scheme:



This indicates the origin of β-hydroxybutyric acid from acetoacetic acid and the possibility of their interconversion. Jowett and Quastel admit the possibility that crotonate may pass through butyrate as an intermediate.

Friedemann [62] has pointed out that the reduction of acetoacetic acid to β-hydroxybutyric acid may be looked upon as a mechanism for replacing a strong acid by a weak one.

Final Stages of Fatty-acid Oxidation. Dakin treated acetoacetic acid with hydrogen peroxide and obtained acetic, glyoxylic, and formic acids and carbon dioxide. However, it is believed that under normal conditions of metabolism the end-products are carbon dioxide and water.

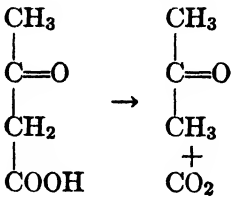
Ketogenesis. Reference has been made to the formation of acetone bodies (*ketogenesis* or *ketosis*) and their excretion in the urine (*ketonuria* or *acetonuria*) in conditions of faulty fat metabolism. Small amounts of these substances appear normally in the urine, particularly

[60] H. D. Dakin, "Oxidations and Reductions in the Animal Body," Longmans, Green & Co., 1922 edition.

[61] *Biochem. J.*, **29**, 2143, 2159, 2773 (1935).

[62] *J. Biol. Chem.*, **116**, 133 (1936).

acetone, which may be regarded as the product of a side reaction.[63] It has its origin in acetoacetic acid as represented by the following equation:



Ketosis occurs in diabetes,[64] in starvation, during the early stages of phosphorus poisoning, during anesthesia, in children during infections, and under other conditions. Acetone bodies increase in the blood and are not only excreted in the urine but appear on the breath as well. When the problem of ketogenesis is examined carefully, it becomes obvious at once that the complete oxidation of fatty acids is in some way dependent on carbohydrate metabolism. In diabetes, the failure to oxidize the fatty acids completely can be related directly to faulty carbohydrate metabolism. When carbohydrate utilization is improved, as by the administration of insulin, the formation of acetone bodies ceases. Similarly, during fasting, as long as glycogen is present, fat metabolism is essentially normal; but just as soon as the reserve carbohydrate is depleted and the starving animal has only fat and protein to draw on for its metabolic needs, the products of incomplete fatty-acid oxidation appear. This does not continue indefinitely, however, for a point may be reached in starvation when, perhaps as a result of some metabolic adjustment, the process of utilizing fat becomes more efficient. The interrelationship between carbohydrate and fat metabolism is indicated by the oft-repeated aphorism, "fats burn in the flame of carbohydrates," and by the following statement of Macleod:

> If the carbohydrate fires do not burn briskly enough, the fat is incompletely consumed; it smokes, as it were, and the smoke is represented in metabolism by the ketones and derived acids.

In addition to the fatty acids certain of the amino acids constitute another potential source of acetone bodies. In conditions of carbohydrate deprivation or in severe diabetes, these substances are ketogenic

[63] For a quantitative study of ketone-body excretion in normal individuals the reader is referred to J. A. Behre (*J. Biol. Chem.*, **92**, 679 [1931]). In 12 subjects, the daily excretion of total acetone bodies (calculated as acetone) varied from 14.5 to 23.5 mg. (average 19.4 mg.), of which 12.2 to 20.5 mg. (average 16.2 mg.) was present as β-hydroxybutyric acid. The maximum excretion during the day occurred in the afternoon or evening and was to a certain extent correlated with an increased urinary volume. Even so short a period of fasting as 6 to 12 hours was accompanied by a small, though unmistakable, rise in the excretion of ketone bodies.

[64] In severe diabetes the daily excretion of acetone bodies may exceed 50 grams. See: G. Lusk, "The Science of Nutrition," 4th edition, 1928, p. 677; P. A Shaffer, "Antiketogenesis," *Harvey Lecture*, 1922–23, p. 105.

(that is, give rise to ketones), as we shall see when we study their metabolism in the next chapter. The liver is believed to be the chief site of the formation of acetone bodies.[65]

Antiketogenesis. In 1921, Shaffer [66] described certain experiments in which he demonstrated that the velocity of oxidation of acetoacetic acid by hydrogen peroxide in an alkaline solution is greatly accelerated in the presence of glucose. The accelerating effect of glucose, thus demonstrated *in vitro*, was accepted as analogous to the well-known fact that, in the body, the oxidation of glucose facilitates the oxidation of fatty acids. This effect, according to Shaffer, may perhaps be explained by assuming the formation of a highly oxidizable compound from acetoacetic acid and some intermediate product of glucose metabolism.

Shaffer's results show that the oxidation of acetoacetic acid occurs only when there is oxidized simultaneously 1 molecule of glucose for each 2 molecules of the acid. The inference is that 2 molecules of acetoacetic acid react with 1 molecule of glucose. It is assumed that 1 fatty-acid molecule gives rise to 1 molecule of acetoacetic acid, and that therefore, in the body, the metabolism of 1 glucose molecule insures the complete oxidation of 2 molecules of fatty acid. Glucose may be replaced by other antiketogenic substances. For example, the antiketogenic value of 1 glycerol molecule is one-half of that of a glucose molecule; from this it follows that a molecule of fat, to burn completely, requires but 1 molecule of glucose. The latter takes care of two fatty acids, while the glycerol makes possible the oxidation of the third fatty acid. Although in the body the antiketogenic or ketolytic value of 1 molecule of glycerol or glyceric aldehyde appears to be equivalent to one-half that of glucose, the behavior *in vitro* is such as to indicate that the ketolytic value of glycerol, glyceric aldehyde, glycol aldehyde, and glyoxal is the same as that of glucose; that is, *in vitro*, a single molecule of any one of these substances will make possible the oxidation of 2 molecules of acetoacetic acid.

The Ketogenic : Antiketogenic Balance in Man. The problem was further extended by Shaffer [67] to the consideration of the relations involved in the ketogenic : antiketogenic balance in man. The substances that may give rise to acetone bodies in metabolism are the fatty acids and certain of the amino acids (leucine, phenylalanine, tyrosine, probably histidine, and possibly others). The substances that have the opposite effect, i.e., the antiketogenic substances, are the carbohydrates, the sugar-forming amino acids, and glycerol.

[65] H. E. Himwich, W. Goldfarb and A. Weller, *J. Biol. Chem.*, **93**, 337 (1931); I. L. Chaikoff and S. Soskin, *Am. J. Physiol.*, **87**, 58 (1928–29); I. A. Mirsky, *ibid.* **15**, 424; **116**, 322 (1936); M. M. Greenberg, *J. Biol. Chem.*, **112**, 431 (1936).

[66] *J. Biol. Chem.*, **47**, 433 (1921); P. A. Shaffer and T. E. Friedemann, *ibid.*, **61**, 585 (1924); Friedemann, *ibid.*, **63**, p. xxi (1925); E. S. West, *ibid.*, **66**, 63 (1925); **74**, p. xlii (1927); see also Hynd, *Proc. Roy. Soc.* (*London*), *B*, **101**, 244 (1927).

[67] *J. Biol. Chem.*, **47**, 449 (1921); **49**, 143 (1921); **54**, 399 (1922).

Considering first the ketogenic factors, it has been estimated that 1 gram of fat (average molecular weight taken as 874) corresponds to 3.43 millimoles of ketogenic material.[68] This is based on the assumption that each molecule of fatty acid gives rise to 1 molecule of acetoacetic acid. Shaffer has estimated the ketogenic equivalent of 1 gram of urinary nitrogen (this represents the metabolism of 6.25 grams of protein) to be 15 millimoles. Accordingly, 1 gram of protein may be expected to yield 2.4 millimoles of acetoacetic acid.

Of the antiketogenic substances, the carbohydrates are most important. One gram of glucose is equal to 5.56 millimoles. For other carbohydrates, it is necessary to calculate first the glucose equivalent. The glucose equivalent of 1 gram of urinary nitrogen (representing the metabolism of 6.25 grams of protein) is 3.6 grams, corresponding to 20 millimoles. Accordingly, 1 gram of protein is equivalent to 3.2 antiketogenic milliequivalents of glucose. One gram of fat contains 1.14 millimoles of glycerol ($1/874 \times 1000$). Since 2 molecules of glycerol are equivalent to 1 molecule of glucose, it follows that the glucose equivalent of 1 gram of fat is 0.57 millimole.

These values are necessary in the calculation of the ketogenic : antiketogenic ratio, R

$$R = \frac{2.4P + 3.43F}{3.2P + 0.57F + 5.56G}$$

In this equation, P is the grams of protein metabolized; F, the grams of fat; G, the grams of glucose. The quantities of these substances burned may be determined either by direct or indirect calorimetry (Chapter XVII). The ratio may also be calculated by substituting, for P, F, and G, the quantities of protein, fat, and carbohydrate, respectively, which are fed. Obviously, this would give, at best, only a rough approximation, owing to the difference between the food fed and the food metabolized. Nevertheless such calculations have been found practically useful in planning dietaries, with the object of either avoiding ketosis, as in the treatment of diabetes, or of producing it, as in the treatment of epilepsy, in which condition beneficial results have been obtained from a ketogenic diet.

In a clinical study of the problem, Woodyatt [69] found that, for the complete oxidation of 1.5 grams of fatty acid, 1 gram of glucose must be utilized. The fatty acid : glucose ratio (FA/G), according to Woodyatt, is therefore 1.5. On a molecular basis, this ratio signifies that 1 molecule of glucose is antiketogenic or ketolytic for 1 molecule of fatty acid. Woodyatt considered this ratio the threshold for ketosis which, when exceeded, would result in acetonuria. Although this observation has

[68] $\frac{3 \times 1000}{874} = 3.43.$

[69] *Arch. Internal Med.*, **28**, 125 (1921).

been confirmed by others, a close study of the problem has disclosed that between *FA/G* ratios (on a molecular basis) of 1 : 1 and 2 : 1 relatively small amounts of acetone are produced. It is only when the 2 : 1 ratio is exceeded that the production of ketone bodies is significant and in general it then corresponds to the excess of fatty acids. This is a remarkable confirmation of Shaffer's theory, based originally on *in vitro* experiments, that the combustion of 1 molecule of glucose permits the simultaneous oxidation of 2 molecules of fatty acid.

Individual variations in susceptibility to ketosis occur. McClellan and associates [70] have observed that in an obese individual the threshold for ketosis was definitely above normal (about 1 : 2.4). This observation has been interpreted as indicative of a greater efficiency in the utilization of fats than in normal men. Perhaps the Eskimos are also better able to utilize fat, as suggested by Heinbecker,[71] who showed that Eskimos excrete but very small amounts of acetone bodies during starvation.[72]

Primates develop fasting ketosis under conditions of metabolism that are qualitatively and quantitatively similar to those which produce ketosis in man.[73] In the rat, the relations are likewise in agreement with Shaffer's theory. The cat and dog, on the contrary, have a high tolerance for ketones, do not develop a starvation ketosis, and dispose of large amounts of acetoacetic acid administered intravenously.

Ketogenesis and Acid-base Balance. The relation of acetone bodies to the acid-base balance of the blood has already been referred to. The acetone bodies are excreted partly in combination with fixed base and partly in combination with ammonia. This accounts both for the depletion of the alkali reserve of the body and for the increased excretion of ammonia in the urine.

The most pronounced manifestations of ketosis are observed in severe diabetes. Values in excess of 300 mg. of acetone bodies per 100 cc. of blood and daily urinary excretions of acetone bodies of 50 grams, or more, have been encountered not infrequently. The acidosis is correspondingly severe. An individual in diabetic coma may

[70] *J. Biol. Chem.*, **80**, 639 (1928).

[71] *Ibid.*, **80**, 461 (1928); **93**, 327 (1931).

[72] Deuel and Gulick (*J. Biol. Chem.*, **96**, 25 [1932]) have made the remarkable observation in human subjects that women develop a much greater ketosis than males during fasting. They suggest that this may be due to either a greater glycogen store in the male or a more economical utilization of the glycogen during the early days of the fast. From a control value of 0.02 gram of acetone bodies, the excretion rose in the male subjects to an average of 1.9 grams on the third day and 2.66 grams on the fourth day. In the females, the initial value was likewise 0.02 gram; it rose to 8.47 grams on the third day, and diminished somewhat on the fourth day to 6.56 grams. Accompanying these changes in the female was a marked acidosis; in one subject the CO_2-combining power diminished from 53.6 on the first day to 26.9 volumes per cent on the fourth day of the fast. In a male subject, the change was much less; 52.1 to 46.6 volumes per cent.

[73] T. E. Friedemann, *J. Biol. Chem.*, **105**, 335 (1934); see also W. Goldfarb, *ibid.*, **116**, 787 (1936).

have a CO_2-combining power of 15 volumes per cent or even less. Both the ketosis and acidosis may be relieved by insulin, or by insulin and glucose, the response being remarkably rapid.

Acidosis accompanies ketosis in starvation. However, neither is very pronounced in male adults; but in women and particularly in children these manifestations may be severe. In carbohydrate deprivation, if the ketogenic : antiketogenic ratio exceeds 2 : 1, ketosis and acidosis develop. The greater tendency of pregnant women to develop ketosis as a result of carbohydrate deprivation has been associated with a lowered glycogen content of the liver.

The ketosis which results from vomiting is essentially of the same type as that occurring in starvation, being due to food deprivation. Accordingly, it is usually most pronounced in children. The formation and excretion of acetone bodies tend to produce acidosis by diminishing the alkali reserve. However, as has been stated elsewhere, if large amounts of gastric contents are vomited, the loss of Cl^- and the corresponding bicarbonate excess tend to produce an alkalosis. In adults, the latter may outweigh the tendency to acidosis, so that it is not uncommon in such cases to find ketosis accompanied by an alkalosis. On the other hand, in children, and especially in infants, the onset of ketosis may be so rapid that it will be accompanied by acidosis. In the toxemias of pregnancy ketosis is very marked. Usually it is associated with acidosis, which may be of only moderate degree, owing to the opposing tendency toward alkalosis resulting from the loss of gastric contents.

Cause of Ketogenesis. We may now turn to a brief consideration of the cause of ketogenesis. It is obvious, of course, that when carbohydrate is not available for metabolism, whether from an insufficient exogenous carbohydrate supply, a deficiency in endogenous reserve, or through the impairment or loss of the ability to oxidize glucose, the organism is forced to burn fat in much larger amounts than normally. The oxidation of fat under these altered conditions becomes the most prominent reaction of metabolism and, as Leathes has expressed it, there is a flooding of the "metabolic mill" with fat. This may conceivably result in the incomplete oxidation of the fat and the production of acetone bodies. There are many examples of chemical reactions in which the intermediate stages can be detected only when one part of a given reaction is exaggerated. For instance, in severe muscular exercise, the formation of lactic acid is more rapid than its removal (either by oxidation or otherwise). The result is that lactic acid accumulates in the blood and a certain amount of it escapes in the urine. Many circumstances suggest a close parallelism between this and ketogenesis, the latter probably being the result of a one-sided metabolism in which excessive amounts of fat are burned. According to this view, the antiketogenic effect of glucose would be due to its fat-sparing action.

Ketosis occurs in rabbits injected with an extract of the anterior lobe of the hypophysis. It is believed to be due, first, to accelerated hepatic glycogenolysis, which in turn leads to a decrease of carbohydrate utilization by the liver cells in the presence of increased fatty-acid catabolism. Ketosis may be abolished under these conditions by checking glycogenolysis through the administration of insulin, or by removal of the liver.[74]

It has been suggested that antiketogenic substances possibly exert their effect by forming easily oxidizable condensation products with acetoacetic acid. Derivatives of glucose and acetoacetic acid, or its ester, prepared by West,[75] have been found to be very unstable. Whether the production of analogous readily utilizable compounds is involved in the mechanism of antiketogenesis is a matter of speculation. There is at present no direct evidence in support of this view; nor is there any direct evidence that the intermediate formation of readily oxidizable condensation products cannot and does not occur (Friedemann).[76]

Fat as a Source of Carbohydrate. The conversion of glycerol into glucose is well established. In the diabetic animal this conversion has been demonstrated by Chambers and Deuel,[77] who administered glycerol to a phlorhizinized dog and recovered 97 to 98 per cent as extra glucose in the urine.

While the conversion of carbohydrate into fatty acids is unquestioned, the reverse transformation of fatty acids into glucose and glycogen is one of the most controversial subjects in physiological chemistry. The opposing points of view have been presented by the late Professor Lusk,[78] who categorically denied the conversion of fat into glucose, and by the late Professor Macleod,[79] who was perhaps the outstanding exponent of the concept that, under certain conditions, if not always, fatty acids serve as a source of carbohydrate. The liver has been discussed as the probable site of this conversion.[80]

The transformation of fatty acids into carbohydrate is believed to occur in the plant during germination. The formation of carbohydrate at the expense of fatty acids in the silkworm has also been described. More recently Gemmill and Holmes [81] compared the respiratory quotient (see p. 515) of liver slices from rats that had been fed (1) a normal diet,

[74] I. A. Mirsky, *Am. J. Physiol.*, **116**, 322 (1936).

[75] *J. Biol. Chem.*, **66**, 63 (1925); **74**, 561 (1927); C. V. Moore, R. J. Erlanger, and E. S. West, *ibid.*, **113**, 43 (1936).

[76] According to Friedemann (*Proc. Soc. Expll. Biol. Med.*, **23**, 370 [1926], *J. Biol. Chem.*, **109**, xxxiv [1935]), only sugars with free "ose" groups (aldose, ketose) are ketolytic. Sucrose is therefore without effect in *in vitro* reactions. See also *J. Biol. Chem.*, **116**, 133 (1936).

[77] *J. Biol. Chem.*, **65**, 21 (1925).

[78] "Science of Nutrition," 4th edition, Saunders, Philadelphia, 1928, pp. 209, 405, 639–643.

[79] "Carbohydrate Metabolism and Insulin," London, 1926, p. 130.

[80] H. Jost, *Z. physiol. Chem.*, **197**, 90 (1931).

[81] *Biochem. J.*, **29**, 338 (1935).

(2) a diet of butter. The average for the first group was 0.79, while for the second group, the respiratory quotient was 0.58, a value consistent with the conversion of fatty acid into carbohydrate. It was also found that the carbohydrate content increased during incubation.

Considering some of the more direct experimental work in mammals, reference may be made to the study of Chaikoff and Weber,[82] who injected repeated doses of epinephrine into depancreatized dogs and found an extra excretion of glucose in the urine, which they could not account for as being derived from either the glycogen store of the liver, glycerol, or tissue protein. According to these investigators, the epinephrine did not produce an increase in the lactic acid of the blood. This point has been disputed, however, by Cori and Cori,[83] and others, who have observed the formation of lactic acid under similar experimental conditions. It has therefore been contended that Chaikoff and Weber did not give due consideration to the muscle glycogen as the indirect source of the extra glucose.

In phlorhizinized dogs, Wertheimer [84] observed an increase in liver glycogen following insulin injection. The more fat contained in the liver, the more resistant did the animals seem to the development of insulin hypoglycemia and the more quickly did the sugar level return to normal. These observations Wertheimer interpreted as evidence for the transformation of fat to glucose and for the idea that insulin accelerates this reaction. These experiments have been repeated by Hawley,[85] whose results in no way indicated that conversion of fat into carbohydrate occurred under these conditions.

Soskin [86] fed depancreatized dogs fat and observed an extra excretion of glucose, the source of which could not be accounted for on the basis of the known precursors from the body. The gluconeogenesis was therefore attributed to fatty acids. This type of experiment has obvious physiological limitations, a fact not unrecognized by Soskin.

A recent report from Deuel's laboratory states that the ethyl esters of the odd carbon fatty acids (propionic to undecylic) give rise to glycogen when fed to fasting rats, while those of the even carbon fatty acids (butyric to myristic and oleic) yield no glycogen.[87]

Low respiratory quotients, below 0.7, have been frequently reported for animals during hibernation. It is stated that during this period of winter sleep the glycogen does not disappear completely and that the nitrogen excretion is too low to make it seem probable that protein is the exclusive source of carbohydrate. The only remaining source of the

[82] *J. Biol. Chem.*, **76**, 813 (1928).

[83] *Ibid.*, **85**, 275 (1929); see also W. H. Bachrach, W. B. Bradley and A. C. Ivy, *Am. J. Physiol.*, **117**, 203 (1936).

[84] *Arch. ges. Physiol.*, **213**, 298 (1926).

[85] *Am. J. Physiol.*, **101**, 185 (1932).

[86] *Biochem. J.*, **23**, 1385 (1929).

[87] J. S. Butts, H. Blunden, W. Goodwin, and H. J. Deuel, *J. Biol. Chem*, **117**, 131 (1937).

carbohydrate would appear to be the fat reserves. Many of these observations have been criticized on the ground of the faulty methods employed in the determination of the respiratory quotient.

A number of years ago Rapport,[88] in his review on the subject of the interconversion of the major foodstuffs, summarized the data that have been offered in support of the fatty acid → carbohydrate transformation and concluded that " no convincing proof exists for the production of carbohydrate from fatty acids in the animal, and that on the contrary, the weight of evidence is at present against it." He nevertheless recognized that the case cannot be considered as closed. This statement still holds.

The Utilization of Fat in the Production of Energy. There can be no doubt of the utilization of fat by the animal body in the production of energy. The evidence for the utilization of fat by muscles is afforded by the experiments of Zuntz,[89] Benedict and Cathcart,[90] Krogh and Lindhard,[91] and others. If muscular work is accomplished at the expense of carbohydrates, the respiratory quotient is high, whereas if fats are burned, the quotient is low. Calorimetry will be considered later in greater detail; for the time being, it is sufficient to state that, by measuring the respiratory quotient of animals during muscular exercise, a fair idea may be had regarding the kind of material which is being burned. This is therefore one method of studying the problem in question.

Blood leaving a working muscle contains less fat than the blood entering it (Lafon).[92] Himwich [93] found a similar relation on comparing the venous and arterial blood of the lower extremity in phlorhizinized or depancreatized dogs. These observations taken in conjunction with the data that have been obtained in determinations of the gaseous exchange of muscle *in situ* and *ex situ* add materially to the chain of evidence that fat oxidation occurs in muscle.

The results of Zuntz and those of Benedict and Cathcart show that fats and carbohydrates are about equally well utilized in the production of muscular work. In fact, it seems that moderate work may be carried on for long periods, with fat as the principal source of energy. However, in the performance of very strenuous, or maximal, muscular work, carbohydrate is essential. Under these conditions there is apparently a selective utilization of carbohydrate. This is partly indicated by the rise of the respiratory quotient. It has also been determined (Chris-

[88] *Physiol. Rev.*, **10**, 349 (1930).

[89] N. Zuntz, "Die Quellen der Muskelkraft," "Oppenheimer's Handbuch Biochem.," **4**, Part I, 826 (1911).

[90] F. G. Benedict and E. P. Cathcart, "Muscular Work," Carnegie Inst. Publ. (1913).

[91] *Biochem. J.*, **14**, 290 (1920).

[92] *Compt. rend. Acad. Sci.*, **156**, 1248 (1913).

[93] H. E. Himwich, W. H. Chambers, A. L. Hunter, and M. A. Spiers, *Am. J. Physiol.*, **99**, 619 (1931–32). Himwich and W. B. Castle, *ibid.*, **83**, 92 (1927); Himwich and M. I. Rose, *ibid.*, **88**, 663 (1929); see also H. B. Richardson, E. Shorr, and R. O. Loebel, *J. Biol. Chem.*, **86**, 551 (1930).

tensen,[94] Dill, Edwards, and Talbott[95]) that individuals performing maximal work reach a state of exhaustion much sooner when on a high fat diet than when on a diet rich in carbohydrate. Christensen's subjects when kept on a high carbohydrate diet lasted 4 hours before breaking down from exhaustion (sore and stiff joints, etc.). Working under the same conditions but fed a high fat diet they lasted only 90 minutes. Prominent among the symptoms which developed were those associated with hypoglycemia; indeed, the demand for carbohydrate was often acute, and the administration of glucose brought prompt relief and enabled the subjects to resume work within a few minutes.

Ketosis has also been observed in persons doing maximal work on a low carbohydrate intake.

Further Observations on the Interchange of Fat in the Animal Body. *Muscular exercise* produces in fasting individuals an increase in the blood lipids, confined largely to the glyceride fraction, suggesting a mobilization of this source of energy from the adipose tissue in response to the demands of the working muscles.[96] In a recent study by Stewart[97] normal men performed muscular work on an ergometer bicycle at rates varying from about 800 to 1200 kg-m. per minute. The blood fat usually rose after about 8000 kg-m. of work had been done. With greater rates of work the increase in blood fat occurred earlier. Continuance of muscular exercise led in time to a return of the blood fat toward normal and later to a second rise. After recovery from a first period of work, a second period produced a rise in the blood fat more easily than usual. When the initial blood fat was abnormally high, as it was in some of the diabetic, as well as normal, subjects, work produced a preliminary fall. On the other hand, in individuals with an abnormally low fasting blood fat, the response was more conspicuous than otherwise and occurred even as a result of relatively light muscular exertion. The increase in blood fat could be abolished by the administration of 100 grams of glucose *per os* prior to the muscular exertion.

The following data illustrate the effect of muscular exercise:

Subject	Work, Kg-m.	Blood Sample	Fat, mg. per 100 cc.	Cholesterol, mg. per 100 cc.	Lipid P, mg. per 100 cc.
REI.....	33,000	Before work	511	118	18.8
		After work	674	118	16.5
		After partial recovery	467	.....	19 0
		After complete recovery	425	.. ...	15.0

[94] *Arbeitsphysiol.*, series of papers in Vols. 4, 5, and 7 (1931–34).

[95] *J. Physiol.*, 77, 49 (1932). The reader is referred particularly to D. B. Dill's review, "The Economy of Muscular Exercise," *Physiol. Rev.*, 16, 263 (1936).

[96] J. W. T. Patterson, *Biochem. J.*, 21, 958 (1927).

[97] C. P. Stewart, R. Gaddie, and D. M. Dunlop, *ibid.*, 25, 733 (1931).

Lymph in the Transportation of Fat. In considering the transportation of fat in the body and particularly the mobilization of fat from the depots, attention in the past has been primarily directed toward the changes produced in the blood, the lymph having been principally associated with the transportation of fat absorbed from the intestine. It now appears from the work of Ivy and associates [98] on fasting normal and phlorhizinized dogs that the lymph also participates in the transportation of reserve fat to the tissues.

Pregnancy. As regards fat metabolism, the embryo is in a large measure independent of the maternal organism. It has even been stated that the placenta does not permit the passage of fat from the maternal to the fetal circulation, the evidence for this being that the lipid content of the maternal blood is higher than that of the fetal blood (Slemons and Stander [99] and others [100]). However, such findings may also signify removal of fat by the fetal organism. This is the conclusion reached by Boyd and Wilson [101] on the basis of chemical analysis of the blood obtained from the umbilical vein and artery.

The way in which fetal metabolism may diverge from the metabolism of the maternal organism is exemplified by the observations of Wesson,[102] who fed pregnant rats diets containing 5 per cent of either butterfat or cod-liver oil and compared the composition of the maternal and fetal tissues with respect to their content of the highly unsaturated fatty acids. The maternal tissues were markedly affected by the two diets. In proportion to the total fat present, the unsaturated fatty acids on the cod-liver oil rations were nearly four times as abundant as on the butterfat diet. In contrast was the close similarity in the ratios of unsaturated fatty acids : total fat in the fetuses of the two groups of rats. Of several possible explanations, Wesson considered the most likely to be that the fetal organism possessed the capacity of synthesizing its own particular type of fat.

In a more extensive study, Chaikoff and Robinson [103] compared the effects of coconut oil, butter, Crisco, peanut oil, cottonseed oil, corn oil, and linseed oil, fats having iodine numbers ranging from 8 to 179. The fats constituted 40 per cent of the weight of the diet. The result of feeding these different rations was a marked variation in the composi-

[98] H. R. Rony, B. Mortimer, and A. C. Ivy, *J. Biol. Chem*, **96**, 737 (1932).

[99] *Bull. Johns Hopkins Hosp.*, **34**, 7 (1923); see also J. R. Murlin, *Am. J. Obstet.*, **75**, 913 (1917).

[100] Mendel and Daniels, *J. Biol. Chem.*, **13**, 71 (1912–13), in feeding experiments with fats treated with fat-soluble stains (Sudan III, Biebrich Scarlet, etc.) found that the placental membrane was impermeable to the ingested fats. On the other hand, Hofbauer (cited by J. Needham, "Chemical Embryology," Vol. II, p. 1192) fed coconut oil to pregnant dogs and reported finding lauric acid, a constituent of the oil, in the fat of the fetus, indicating its placental transmission.

[101] *J. Clin. Investigation*, **14**, 7 (1935); E. M. Boyd, *Biochem. J.*, **29**, 985 (1935).

[102] *Bull. Johns Hopkins Hosp.*, **38**, 237 (1926).

[103] *J. Biol. Chem.*, **100**, 13 (1933).

tion of the maternal fat, as indicated by the data in Table XLIX. In the fetuses, the quality of the fat was not influenced to the same degree, being modified only about one-fifth as much as the maternal fat. The extent to which the fetal fat was thus modified was more closely related to the degree of unsaturation of the fat of the mother than that of her diet.

TABLE XLIX

EFFECT OF FOOD FAT ON FETAL AND MATERNAL FAT

Diet	Average Iodine Number		
	Diet	Maternal Fat	Fetal Fat
Coconut oil	8 0	41.1	68 0
Butter	33 0	57.8	70 5
Crisco	75 0	79 0	76 8
Peanut oil	95 0	94 0	80 0
Cottonseed oil	107 0	102 0	81 8
Corn oil	124 0	117 0	81 6
Linseed oil	179 0	139 0	88 2
Starch (fat-poor diet)		65 0	72.5
Protein (fat-poor diet)		66 7	74 1

During pregnancy, and particularly in the later stages, the lipid content of the blood is conspicuously elevated. The lipemia is due almost entirely to the increase in plasma lipids, with very little change in the corpuscles. The neutral fat begins to increase during the first trimester and attains at term a concentration which is about twice the non-pregnant value. The phospholipid and cholesterol fractions increase moderately, the concentrations at term being about 20 to 25 per cent above the non-pregnant values.[104]

Lactation. It is possible that the fat mobilized at term by the maternal organism not only provides the fetus with an extra supply as evidenced by an extra deposit in the fetal livers, but that a portion is also utilized by the mother in the production of colostrum. After parturition, the concentrations of the various blood lipids continue to be high. In cattle, Maynard and associates [105] have observed a sharp rise after delivery, the increase being maintained for a considerable part (140 days) of the lactation period. Meigs, Blatherwick, and Cary[106] considered the milk fat to be derived from the phospholipids of the blood. A more recent study of the problem has disclosed that blood entering the mammary

[104] E. M. Boyd, *J. Clin. Investigation*, **13**, 347 (1934).

[105] L. A. Maynard, E. S. Harrison, and C. M. McCay, *J. Biol. Chem.*, **92**, 263 (1931); McCay and Maynard, *ibid.*, **92**, 273 (1931); *J. Nutrition*, **2**, 67 (1929–30).

[106] *J. Biol. Chem.*, **37**, 1 (1919).

gland (internal iliac artery) has essentially the same phospholipid content as the blood leaving it (mammary vein). Nor is there any significant difference in regard to cholesterol, but the total fatty-acid concentration is consistently higher in the artery than in the vein. These findings indicate that blood fat rather than the phospholipid fraction is utilized in the production of milk fat.[107] Indeed, there seems to be a correlation between the amount of blood fat and the quantity of milk secreted. Late in lactation, accompanying the reduction in blood lipids is a marked diminution of milk production, and hence of the total fat secreted, but the concentration of the fat in the milk remains relatively unchanged. In cattle maintained on rations low in fat the same correlation has been observed, namely low blood lipids and diminished milk formation, but no change in the fat content of the milk. Such information as is available at present indicates that the fat of the diet, as well as the reserve fat of the tissues and the fat which the maternal organism is capable of synthesizing, are the precursors of the milk fat. That the mammary gland does not merely secrete the fat of the maternal blood, but also plays a definite rôle in the production of a more or less characteristic fat from these precursors, is indicated by the work of Petersen, Palmer, and Eckles.[108] The occurrence in milk fat of so large a variety of fatty acids, ranging in size from C_4 to C_{26}, is of particular interest.[109]

Metabolism of Cholesterol. The synthesis and destruction of cholesterol in the animal organism has been demonstrated by many investigators. For example, it has been shown by Schoenheimer and Breusch [110] that mice fed a diet low in sterol synthesized an average of 1.8 mg. of cholesterol daily. When moderate quantities of cholesterol were added to the diet, a smaller amount of cholesterol was synthesized, and when large amounts of cholesterol were fed, a considerable part was destroyed. These experiments showed that cholesterol is synthesized when the demand exceeds the supply and is destroyed when the supply exceeds the demand.

Rittenberg in Schoenheimer's laboratory has determined that mice fed deuterium-containing food over a period of time synthesize cholesterol that contains a certain amount of deuterium. This confirms the view that cholesterol may be formed from numerous units of small molecular weight.

Aside from its importance as an essential constituent of cells, cholesterol is of extraordinary interest because of its close chemical relation to such physiologically important substances as the bile acids, vitamin D, and the sex hormones. Sterol metabolism is a many-sided topic concerning which comparatively little is known at present. However,

[107] W. R. Graham, T. S. G. Jones, and H. D. Kay, *Proc. Roy. Soc.* (London) *B*, **120**, 330 (1936).

[108] *Am. J. Physiol.*, **90**, 573 (1929).

[109] G. E. Helz and A. W. Bosworth, *J. Biol. Chem.*, **116**, 203 (1936); T. P. Hilditch, *et al.*, *Biochem. J.*, **30**, 677, 1905 (1936).

[110] *J. Biol. Chem.*, **103**, 439 (1933).

certain observations have been made which may be regarded as significant. It has been determined that cholestenone yields either cholesterol or coprosterol, depending on whether the animal (dog) is on a low or high meat diet. The conversion of coprostanone to coprosterol (in man and dog) has also been established. It would therefore seem that the change from cholesterol to coprosterol involves the intermediate formation of cholestenone and coprostanone. This seems all the more probable since the occurrence of allocholesterol, another possible intermediate, has been ruled out.[111]

The dependence of cholesterol metabolism on thyroid function is indicated by the marked increase in blood cholesterol (hypercholesterolemia) in conditions of hypothyroidism and the tendency to low values in hyperthyroidism. Hypercholesterolemia occurs also in diabetes, chronic hemorrhagic nephritis, and especially in so-called nephrosis.

Normally about 75 per cent of the plasma (or serum) cholesterol is present in combination as esters. Sperry [112] has shown that when sterile serum is incubated a proportion of the free cholesterol is esterified. According to Sperry this signifies that an active cholesterol esterase is present in blood serum and that the combined and free cholesterol are not in equilibrium. Apparently the point of equilibrium lies in the direction of an increased proportion of combined to free cholesterol.

Esterification of cholesterol probably occurs in various tissues (intestinal mucosa, etc.), but the importance of the liver as a site of this reaction is revealed by the fact that in diseases involving this organ, as well as in obstruction of the biliary tract, the proportion of cholesterol in ester combination is markedly reduced, while the amount of free cholesterol is correspondingly increased.

A relation of cholesterol to the structure and properties of muscle is suggested by Bloor,[113] who analyzed the three types of muscle (smooth, cardiac, and skeletal) of various mammals, birds, and cold-blooded animals. Smooth muscle was found to have the highest cholesterol content (average of 0.77 per cent on the basis of dry weight of the muscle). Cardiac muscle was next with 0.55 per cent, and skeletal muscle was lowest with about 0.3 per cent as the average. Smooth muscle, on the other hand, was found to have the lowest phospholipid/cholesterol ratio (average 4). For cardiac and skeletal muscle this ratio was roughly four times as much. Bloor points out the possibility that these peculiarities in chemical composition may be significant; that to some extent the cholesterol content may be related to spontaneous activity of smooth and cardiac muscle, and the phospholipids to energy expenditure.

Products of Fat Anabolism and Catabolism. Like other foodstuffs, the fats have a dual fate in metabolism. In addition to their utilization in the production of energy, a portion is used in the synthesis of essential

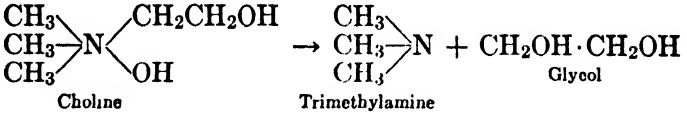
[111] R. Schoenheimer, D. Rittenberg and M. Graff, *J. Biol. Chem.*, **111**, 183 (1935).
[112] *J. Biol. Chem.*, **111**, 467 (1935).
[113] *J. Biol. Chem.*, **114**, 639 (1936).

tissue constituents many of which have been considered in an earlier chapter. The phospholipids and cerebrosides are synthesized *de novo* in the body, and it appears probable that the organism is also capable of producing cholesterol and perhaps other sterols. It is to some of these constituents that the permeability and other physical properties of cells are partly attributed. The part played by fats and fat-like substances in the life of the cell has been reviewed by Leathes.[114]

The lipids secreted by the sebaceous glands (sebum) enable certain animals, particularly the fur- and feather-bearing ones, to shed water. The secretions on the skin also serve to diminish heat radiation. Sebum is a mixture of a number of fatty substances and is secreted by the sebaceous glands of the skin. A similar substance is cerumen, which is formed in the sebaceous and sweat glands of the cartilaginous part of the outer ear.

Lipoproteins are present in the blood as well as in many tissues. Taylor [115] has stated that, if a gland like the kidney which is rich in lipoproteins be completely extracted with fat solvents and then digested with trypsin, a subsequent extraction will yield a goodly amount of fatty substance. Some of this will be found to consist of phosphatides and sterols and the remainder of neutral fat.

Lipoproteins are very probably broken down into their respective lipid and protein residues. It is assumed that these follow the usual paths of protein and fat metabolism. The lecithin is presumably broken down to glycerol, fatty acids, phosphoric acid, and choline. The last-named substance is believed to give rise to trimethylamine, small amounts of which occur in urine. The formation of trimethylamine is represented by the following equation:



[114] J. B. Leathes and H. S. Raper, "The Fats," Chapter X, 1925 edition; also Leathes in *Lancet*, 1, pp. 803, 853, 957, 1019 (1925).

[115] A. E. Taylor, "Digestion and Metabolism," 1912, p. 347.

CHAPTER XIII

INTERMEDIARY METABOLISM OF PROTEIN

There is a continuous and relatively uniform degradation of protein in the animal body, which is often described as the " wear and tear " of the tissues. Obviously this loss must be restored, as otherwise the animal would gradually waste away. The formation of proteins characteristic of a particular tissue apparently depends on the availability in sufficient amounts of the necessary amino acids. Such indeed is the destiny of a considerable proportion of the amino acids derived from the proteins of the diet. A second fate of amino acids in metabolism is their utilization for some specific purpose, such as the formation of hormones, bile salts, catalysts, purines, pigments, etc. As has been pointed out by McCance,[1] the number of reactions in this class is large, and each one is a law unto itself. Inasmuch as there is ordinarily no storage of protein in the body of an adult animal,[2] the actual protein needs are not in excess of the amount required to replace the protein lost and for the specific reactions just mentioned. However, if the protein intake is limited to these requirements and if the supply of carbohydrate and fat is inadequate to meet the caloric needs of the animal, the body may be forced to depend on the protein of its own tissues for the supply of energy. At such times an excessive amount of tissue breakdown occurs.

[1] R. A. McCance, "The Chemistry of the Degradation of Protein Nitrogen," *Physiol. Rev.*, **10**, 1 (1930).

[2] This statement is based on the observation that the normal, well-nourished adult readily maintains nitrogen equilibrium on a moderate intake of protein. The possibility of protein storage at least temporarily is not excluded: (1) in the undernourished individual placed on a liberal protein intake, (2) in the well-nourished individual on excessive protein diet. Indeed, recent experimental evidence supports the view that the organism holds in reserve considerable amounts of protein which are depleted when the supply is cut off. Moreover, the protein reserve may be materially influenced by the level of protein intake. In the albino rat, for example, the importance of the liver as a reservoir of protein is revealed by the fact that, during a two-day fast, 20 per cent of the liver protein disappears, while the kidneys and other organs lose only 4 per cent of their protein. Addis, Poo, and Lew, *J. Biol. Chem.*, **115**, 111, 117 (1936), found that, in longer periods of starvation, the liver lost 40 per cent of its protein, this representing 16 per cent of the total depletion. Muscle, skin, and skeleton contributed 62 per cent, the kidneys 1 per cent, the heart 0.5 per cent, and the adrenals and testes nothing.

The influence of a high protein diet in augmenting the content of the various protein fractions in the liver has been described by Luck, *J. Biol. Chem.*, **115**, 491 (1936).

It is evident that if the amino acids of the proteins of the diet are not present in the same proportions as in the proteins of the animal, there must be an excess of some amino acids which are not utilized in protein synthesis and hence must be disposed of in some other way. Moreover, the protein intake is usually in excess of the anabolic needs of the organism. Consequently, a certain amount of the amino acids derived from the diet is burned directly or converted into sugar and fat. *Exogenous metabolism* is the metabolism of all protein ingested in excess of that required by the tissues for maintenance and growth. *Endogenous metabolism*, on the other hand, usually refers to the metabolism which produces as end-products creatinine, neutral sulfur, and the part of the urea and uric acid not derived from the food.[3]

Nitrogen Equilibrium. By comparing the intake of nitrogen as protein with the total elimination of nitrogen (in the urine, feces, and perspiration), it is possible to determine whether the body is gaining or losing protein. If such studies are carried on over a short period, such as 24 hours, there may appear to be a retention of nitrogen. This is more often due to a lag in the elimination of the nitrogenous end-products of protein-metabolism than to a synthesis of protein. For accurate work, studies in nitrogen equilibrium should be carried on over a period of several days, separate analyses being made of the food and excreta of each day. In the adult, there is ordinarily no retention of nitrogen; that is, the nitrogen intake as protein is equivalent to the nitrogen elimination. This is a condition of *nitrogen equilibrium*, or *nitrogen balance.*

The statement that in the adult individual there is no retention of nitrogen needs to be qualified. It is well known that muscular development and a gain in weight result when an individual performs muscular work over a period of several weeks or months, provided he is supplied during this time with an adequate amount of food, especially protein. Bornstein [4] investigated this question and found that nitrogen retention occurred (positive nitrogen balance) when the increase in muscular activity was accompanied by an increase in the protein intake.

Negative nitrogen balance occurs when the endogenous protein metabolism exceeds the protein intake. This occurs in malnutrition, starvation, fevers, and other wasting diseases. In fevers, the patient is often given large amounts of sugar for the purpose of sparing the tissue proteins as much as possible. The more essential organs, such as the heart and brain, are spared even in prolonged starvation. During convalescence there is regeneration of the tissues and hence a retention of nitrogen. Conservation of body protein and its utilization in the production of hemoglobin has been observed in patients recovering from

[3] For an excellent summary of the theories of endogenous and exogenous protein metabolism the student is referred to H. H. Mitchell and T. S. Hamilton, "The Biochemistry of Amino Acids," Chemical Catalog Co., New York, 1929, Chapter IX.

[4] *Arch. ges. Physiol.*, **83**, 540 (1901).

anemia as a result of effective therapy (iron in hypochromic anemia; liver extract in pernicious anemia).

In growing animals the excretion of nitrogen is less than the corresponding protein intake, a portion of the amino acids of the diet being used in the synthesis of new tissue protein.

Anabolism. Present knowledge of this aspect of protein metabolism is exceedingly vague, resting as it does on a few generalizations and very little, if any, direct experimental evidence. It is generally assumed that amino acids are the building stones in the enzymic synthesis of body protein. The principal source of the amino acids is the protein of the diet, but certain of the amino acids may be synthesized in the body, if the need arises; other amino acids the organism is unable to synthesize. The problems arising from these considerations, such as the nature of the indispensable amino acids, the biological value of different proteins, and the protein requirement in nutrition, will be considered in a later chapter.

A novel viewpoint concerning the synthesis of proteins *in vivo* has been recently advanced by Alcock.[5] Considering the evidence for protein anabolism from amino acids as circumstantial at most, and on the whole inconclusive, he has proposed the theory that the substrate for body protein synthesis consists, not of amino acids, but of simpler nitrogenous units, derived from the amino acids.[6] Alcock has made the startling assumption that " the amino acid groups appear for the first time in the proteins they ultimately comprise, and the composition of the feeding medium is with certain reservations immaterial."

As regards the essential amino acids, Alcock's contention is that the effects of deficiency of one or more of these (failure to grow, or even loss of weight) need not necessarily be associated with limitation of protein synthesis; another explanation may be invoked, namely, that the missing amino acid has some other function in the organism which accounts for its indispensability.

Alcock's theory, although without proof, is nevertheless stimulating for the reason that it forcefully brings to the attention the lack of direct evidence in support of the prevailing view that amino acids are the units in the anabolism of body protein.

Catabolism. When tissues break down, the protein molecules are apparently hydrolyzed through the action of tissue enzymes, yielding mainly amino acids. There is no reason for assuming that the metabolism of these differs from that of the amino acids derived directly from the diet.

The study of intermediary protein metabolism is essentially the study of the fate of the individual amino acids. This is the main purpose of the present chapter.

[5] *Physiol. Rev.*, **16**, 1 (1936).

[6] Alcock admits the possibility that the nitrogen must be in the form of amino groups.

Metabolism of Glycine. The metabolism of this amino acid may be considered from several angles, the first being that of its breakdown to NH_3, CO_2, and H_2O in normal metabolism. Its conjugation with benzoic acid and other aromatic acids to form hippuric acid and related compounds is to be considered as a phase of its metabolism, as is also the formation of glycocholic acid by conjugation with cholic acid. We also have to include the synthesis of sugar from glycine in the diabetic animal as well as its possible formation in the normal individual.

The steps in the breakdown of glycine in the course of normal metabolism are not definitely known. However, the following changes seem possible:

$$\begin{matrix} CH_2NH_2 \\ | \\ COOH \end{matrix} \rightarrow \begin{matrix} CH{=}NH \\ | \\ COOH \end{matrix} \rightarrow \begin{matrix} C\begin{matrix} OH \\ {-}NH_2 \\ H \end{matrix} \\ | \\ COOH \end{matrix} \longrightarrow NH_3 + 2CO_2 + H_2O$$

In liver perfusion experiments with glycine ammonia is formed.[7] Small amounts of methylamine are also produced, probably by simple decarboxylation:

$$\begin{matrix} CH_2NH_2 \\ | \\ COOH \end{matrix} \rightarrow CH_3NH_2 + CO_2$$

Evidence for the synthesis of glycine in the body is to be found in the numerous experiments in which benzoic acid or benzoates have been fed to animals and hippuric acid found in the urine.[8] Hippuric acid (benzoyglycine) is a normal constituent of the urine of horses, cattle, and other herbivorous animals. In small amounts, it is likewise present in human urine. Not only does the body use any preformed glycine that may be present either in the diet or in the tissues, but it is at times forced to synthesize this amino acid in large amount for the purposes of detoxication. Quick [9] has estimated that the maximum synthesis of glycine in man is 9 mg. per kg., per hour. Although there is no doubt that this occurs, we do not know how it is brought about. Knoop as well as Dakin [10] has suggested that the formation of glycine in the body may result from the oxidation at the β-carbon atom of α-amino β-hydroxy acids such as serine. Dakin has shown that phenylserine does not behave like phenylalanine in the body, for it yields

[7] A. Bornstein, *Biochem. Z.*, **212**, 137 (1929); **214**, 374 (1929); R. Kohn, *Z. physiol. Chem.*, **200**, 191 (1931).

[8] See, for example, the recent papers of W. H. Griffith and H. B. Lewis, *J. Biol. Chem.*, **57**, 1 (1923); Griffith, *ibid.*, **69**, 197 (1926); **82**, 415 (1929).

[9] *Ibid.*, **92**, 65 (1931).

[10] H. D. Dakin, "Oxidations and Reductions in the Animal Body," 1922 edition, Chapter III.

benzoic acid. Thus, it appears to be oxidized at the β-carbon atom as represented in the equation:

$$C_6H_5 \cdot CHOH | CHNH_2 \cdot COOH + O \rightarrow C_6H_5 \cdot COOH + CH_2NH_2 \cdot COOH$$

The possibility of conversion of glycine into glucose in the animal body was demonstrated by Ringer and Lusk.[11] A phlorhizinized dog, having a D : N ratio of 3.38 (see p. 341) was given 20 grams of glycine, and the urine collected during the next 14 hours was analyzed and found to contain 47.42 grams of glucose and 12.84 grams of nitrogen. Since 20 grams of glycine contain 3.73 grams of nitrogen, the difference between 12.84 and 3.73, or 9.11 grams, represents the protein metabolism during this interval. Therefore, $9.11 \times 3.38 = 30.79$ grams of glucose had its origin in the protein metabolized. The difference between 47.42 grams, the total sugar excreted, and 30.79 grams, that is, 16.63 grams, must have been formed, therefore, from the ingested glycine. This accords well with the calculated yield (16 grams) based on the assumption that all of the carbon of glycine is converted into glucose. This conversion may be represented as follows, though actually the path of conversion of glycine into glucose is still obscure:

$$3\ \underset{\text{Glycine}}{CH_2NH_2 | COOH} \rightarrow 3\ \underset{\text{Glycolic acid}}{CH_2OH | COOH} \rightarrow 3\ \underset{\text{Glycolic aldehyde}}{CH_2OH | CHO} \rightarrow \underset{\text{Glucose}}{C_6H_{12}O_6}$$

If this represents the sequence of events, glycolic acid and aldehyde should yield glucose when given to diabetic animals. Sansum and Woodyatt [12] showed that this occurs. As much as 75 per cent of the glycolic acid which they had administered slowly to a phlorhizinized dog escaped oxidation and appeared in the urine as extra glucose.

In contrast to the ready and complete (100 per cent) transformation of glycine into glucose observed by Ringer and Lusk [11] in the diabetic animal is the slight effect in the normal animal. Wilson and Lewis [13] found that feeding glycine to rats produced no change in the glycogen content of the liver, or other tissues, whereas the administration of comparable amounts of *d*- or *dl*-alanine resulted in a definite increase in the glycogen reserves of the body. Butts, Dunn, and Hallman,[14] on the other hand, obtained definite evidence of glycogen formation from glycine fed to rats following a 48-hour fast. The average increase in liver glycogen was, however, only one-fifth of that obtained on feeding *d*-alanine. Glycine also proved to be anti-ketogenic (rats received

[11] G. Lusk, "The Science of Nutrition," 4th edition, 1928, p. 228; Ringer and Lusk, *Z. physiol. Chem.*, **66**, 106 (1910).

[12] *J. Biol. Chem.*, **17**, 521 (1914).

[13] *J. Biol. Chem.*, **85**, 559 (1929–30).

[14] *Ibid.*, **112**, 263 (1935); see also *ibid.*, **119**, 247, **120**, 289 (1937).

acetoacetate), which may be construed as further evidence of its conversion into carbohydrate, but in this respect, too, *d*-alanine was definitely superior to glycine.

Metabolism of Alanine; General Course of Amino-acid Metabolism. The opinion generally held is that oxidation of amino acids occurs in the α position and is accompanied by the removal of the amino group. Much attention has been devoted to the problem in an attempt to determine the intermediate steps of the deamination process. A convenient method for studying this reaction has been developed by Krebs [15] and used by many other workers. It consists in determining the changes (oxygen utilized, production of ammonia, keto acids, etc.) by slices of fresh liver, intestine, kidney, and other organs kept in appropriate nutritive solutions to which is added the amino acid under investigation. It has been found that the kidney and liver contain at least two distinct enzyme systems, one catalyzing the deamination of the natural amino acids, the other acting on the non-natural optical isomerides (Krebs). It appears that the unnatural isomers are preferentially oxidized (rat kidney slices, Krebs; purified preparations from the kidney of various animals, Bernheim and Bernheim [16]).

By the use of the surviving tissue slice method, as well as tissue enzyme preparations, important data have been obtained concerning the deamination process. However, it has not been established with certainty that the reactions observed *in vitro* are identical with those that occur *in vivo*.

It is of historical interest to recall two of the older theories of deamination. Neuberg and Langstein [17] fed a starving rabbit alanine and obtained lactic acid in the urine. Embden [18] perfused a surviving liver with alanine and likewise obtained lactic acid. It was therefore supposed that the formation of α-hydroxy acids was the immediate result of hydrolysis as represented by the following equation:

$$\begin{array}{l} R \\ | \\ CH_2 \\ | \\ CH\cdot NH_2 \\ | \\ COOH \end{array} + HOH = \begin{array}{l} R \\ | \\ CH_2 \\ | \\ CH\cdot OH \\ | \\ COOH \end{array} + NH_3$$

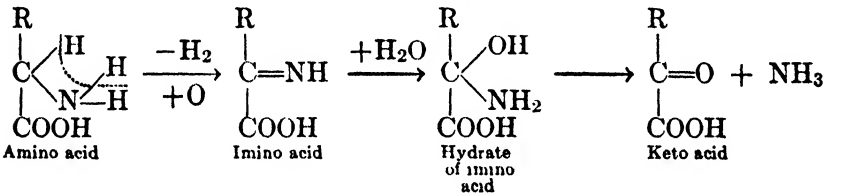
This view has been abandoned since the weight of evidence points to an oxidative deamination with the intermediate formation of α-ketonic acids. In general, α-amino acids and the corresponding α-ketonic acids share the same fate in metabolism and are oxidized with approximately

[15] *Z. physiol. Chem.*, **217**, 191 (1933); *Biochem. J.*, **29**, 1620 (1935).
[16] *J. Biol. Chem.*, **109**, 131 (1935).
[17] *Arch. Anat. Physiol.*, Physiol. Abt., 514 (1903).
[18] Cited by Dakin in "Oxidations and Reductions in the Animal Body," 1922 edition, p. 67.

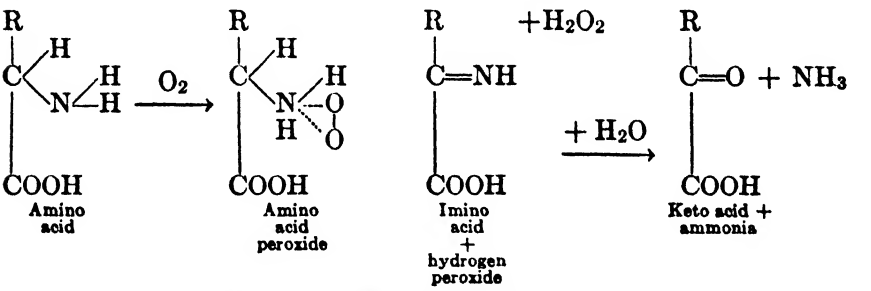
the same rapidity, whereas the α-hydroxy acids are oxidized less readily. It is considered that the α-hydroxy acids are not formed directly from amino acids, as was formerly supposed, but from the corresponding ketonic acids.

The principal sites of amino acid oxidation are the liver, kidney, and intestinal mucosa, in the order named.[19] There are exceptions, as for example tryptophane and histidine, which are little affected by the kidney. In general the simpler amino acids are more readily catabolized (tissue slice method). The exception is glycine, which is relatively resistant to deamination.

According to Knoop,[20] hydrates of imino acids are formed in the process of deamination of amino acids. This is very suggestive, for we may then assume the following sequence of events: The first step in the metabolism of an amino acid may be taken to be one involving the loss of hydrogen by dehydrogenation, resulting in the formation of the corresponding imino acid. Presumably the next step is one of hydration; the hydrate thus formed, because of its instability, parts with its ammonia, yielding a ketonic acid. These changes are represented as follows:



The possibility of formation of amino acid-peroxide compounds, suggested by the work of several investigators,[21] is considered by Krebs [22] of sufficient significance to justify, at least tentatively, the following formulation for the intermediate steps in deamination:



[19] M. Neber, *Z. physiol. Chem.*, **240**, 59 (1936); compare with E. S. London, *et al.*, *ibid.*, **227**, 223 (1934).

[20] *Z. physiol. Chem.*, **67**, 489 (1910); see also F. Knoop and H. Oesterlin, *ibid.*, **148**, 294 (1925), **170**, 186 (1927).

[21] F. Bergel and K. Bolz, *Z. physiol. Chem.*, **215**, 25 (1933); **220**, 20 (1933); **223**, 66 (1934); D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (*London*), *B*, **119**, 114, 141, (1936).

[22] *Ann. Rev. Biochem.*, **5**, 247 (1936).

Fate of Ammonia. The formation of urea and its physiological significance will be considered in detail later. For the present it is sufficient to state that in mammals, and particularly in man, the ammonia which is formed in the deamination of the amino acids is converted almost quantitatively into urea. This is the principal end-product of protein metabolism, the quantity present in the urine being determined largely by the amount of protein ingested. The utilization of ammonia in neutralizing acid will be considered in a later section.

Conversion into Glucose. Alanine may be completely converted, carbon for carbon, into glucose in phlorhizin diabetes, as shown by Ringer and Lusk [11] and by Dakin and Dudley.[23] The occurrence of this reaction in the normal animal is indicated by the results of Wilson and Lewis, and Butts and associates (p. 379). It seems that only *d*-alanine is an effective glycogenic substance, while *l*-alanine is relatively ineffective in this regard.

Fate of Keto Acid. The reactions which follow deamination differ with different amino acids, and as will be seen are not fully understood. According to Krebs [22] the three possible pathways are:

(*a*) Reduction to the hydroxy acid.
(*b*) Resynthesis to the amino acid.
(*c*) Breakdown to a fatty acid with one less carbon atom and subsequent oxidation.

The last represents a step in the continued catabolism of the amino acid. The probable reaction is oxidative decarboxylation, by which is meant simultaneous decarboxylation and oxidation of the aldehyde group (Krebs). The combined reaction may be represented as follows:

$$R\cdot CO\cdot COOH \xrightarrow{+O} R\cdot COOH + CO_2$$

Synthesis of Alanine. Embden and Schmitz [24] reported the synthesis of alanine in a series of experiments in which ammonium pyruvate was perfused through the liver. Alanine was recovered in the perfusate.

Metabolism of Serine. One known fact concerning the metabolism of *l*-serine is its conversion into glucose in the diabetic animal. Oxidative deamination to the corresponding keto acid, $CH_2OH\cdot CO\cdot COOH$ (α-keto, β-hydroxypropionic acid), has been demonstrated for the *dl* form.[25]

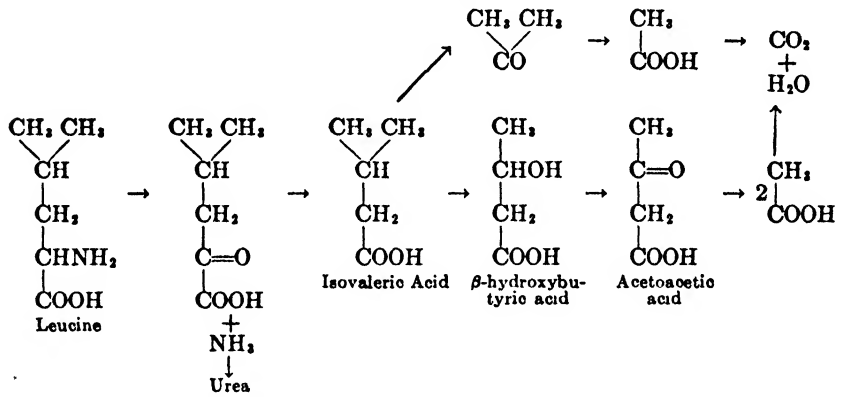
The close chemical relationship of serine and alanine suggests the possibility that these amino acids may be interchangeable in metabolism. The ability of the organism to synthesize serine is suggested by the fact

[23] *J. Biol. Chem.*, **17**, 451 (1914).
[24] *Biochem. Z.*, **38**, 393 (1912); see also M. Neber, *Z. physiol. Chem.*, **234**, 83 (1935).
[25] F. Bernheim, M. L. C. Bernheim, and A. G. Gillaspie, *J. Biol. Chem.*, **114**, 657 (1936).

that growth of the white rat is not affected adversely by its complete exclusion from an otherwise adequate diet.[26]

Metabolism of Valine. The fate of *d*-valine in metabolism is only partly known. Deamination and oxidation at the α-carbon atom yields the corresponding keto acid (Krebs, Neber). It is apparently neither glycogenic nor ketogenic.

Metabolism of Leucine, Norleucine, and Isoleucine. In all three amino acids, oxidation probably conforms to the general course previously outlined. Considering leucine, it is converted to the corresponding keto compound, which is in turn oxidized to isovaleric acid. This is believed to undergo demethylation, forming β-hydroxybutyric acid. From this point, its fate is presumably identical with that of all other fatty acids, oxidation occurring at the β-carbon atom. Hence, leucine may be a source of acetone bodies in the diabetic animal. A certain amount of acetone may be formed directly by the oxidation of isovaleric acid. These reactions are represented below:



Womack and Rose [27] have shown that leucine and isoleucine are indispensable amino acids, while norleucine does not seem to be essential for growth.

A recent study of the glycogenic and ketogenic properties of the three amino acids may be summarized as follows: [28]

[26] R. H. McCoy and W. C. Rose, *ibid.*, **117**, 581 (1937).

[27] *J. Biol. Chem.*, **116**, 381 (1936).

[28] J. S. Butts, H. Blunden, and M. S. Dunn, *J. Biol. Chem.*, **120**, 289 (1937). The experimental procedure employed by these investigators is as follows: (1) For evidence of glycogen synthesis (*a*) determination of effect of feeding a given amino acid on the glycogen content of the liver of rats previously fasted 48 hours and a comparison with the glycogen content of the liver of controls; (*b*) determination of antiketogenic effect of amino acids, by comparing output of acetone bodies in the urine of rats given definite amounts of amino acid and sodium acetoacetate with the output of rats receiving only acetoacetate. (2) For evidence of ketogenesis, determination of output of acetone bodies by rats receiving the amino acid (as sodium salt) and comparison with controls.

	Glycogen formation	Acetone body formation
dl-Leucine	−	+
dl-Norleucine	+	+
dl-Isoleucine	small amount	+

Metabolism of Aspartic Acid. The metabolism of this amino acid has not been precisely formulated. According to Needham,[29] aspartic acid when added to minced muscle under anaerobic conditions gives rise to succinic, $COOH \cdot CH_2 \cdot CH_2 \cdot COOH$, fumaric, $COOH \cdot CH : CH \cdot COOH$, and malic, $COOH \cdot CH_2 \cdot CHOH \cdot COOH$, acids. It is logical to assume the formation of ketosuccinic acid, $COOH \cdot CH_2 \cdot CO \cdot COOH$, as an intermediate of metabolism. Judging from Krebs's data, the rate of oxidation of aspartic acid is slower than that of other amino acids.

Ringer and Lusk [30] found that in phlorhizinized dogs the equivalent of three carbon atoms of the four in aspartic acid could be accounted for in the form of extra glucose excreted in the urine.

The removal of aspartic acid, together with the other dicarboxylic amino acids (glutamic and hydroxyglutamic), from hydrolyzed casein does not alter the nutritive value of the resulting material (St. Julian and Rose).[31] With this as the substitute for protein, young rats have been found to grow normally, indicating that the organism is capable of synthesizing the dibasic amino acids, including aspartic.

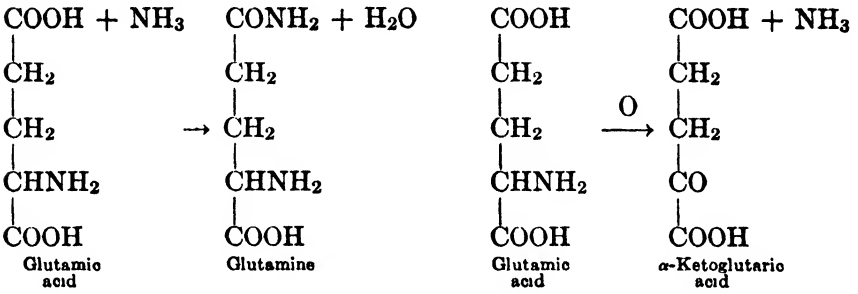
Metabolism of Glutamic Acid. Only three carbon atoms of the five in glutamic acid are converted into glucose in the completely diabetic animal (Lusk).[32] As in the case of aspartic acid, the formation of sugar has been explained in more than one way. Deamination at the α-carbon atom and subsequent oxidation at the β-carbon atom would yield glyceric acid. This, in turn, would be oxidized completely in the normal animal, or it would be converted into sugar, carbon for carbon, in the completely diabetic animal. On the other hand, it is more probable that ketoglutaric acid is formed first, and that this is converted into either malic or succinic acid. Needham [29] has shown that, under anaerobic conditions, succinic acid is formed from glutamic acid when the latter is added to minced muscle. Both malic and succinic acids are non-toxic and presumably are oxidized in the body.

Synthesis of glutamine from glutamic acid and ammonia (ammonium glutamate) is produced by kidney (rabbit, guinea pig), brain, and retina. According to Krebs,[33] relatively large amounts of ammonia may be bound in this manner. The synthesis is enzymic, associated with " glutaminase " which is physiologically inhibited so that reversal of the

[29] *Biochem. J.*, **24**, 208 (1930).
[30] G. Lusk, "The Science of Nutrition," 1928 edition, p. 242.
[31] *J. Biol. Chem.*, **98**, 439 (1932).
[32] *Am. J. Physiol.*, **22**, 174 (1908).
[33] *Biochem. J.*, **29**, 1951 (1935).

reaction does not occur. It is known that brain tissue can utilize glutamine without splitting to glutamic acid and ammonia. Aqueous extracts of the tissues are capable of splitting glutamine to glutamic acid and ammonia. That the enzyme responsible for the synthesis is identical with the hydrolyzing enzyme is suggested by the inhibition of both processes in the presence of the unnatural *d*(−)-glutamic acid.[34]

According to Malherbe,[35] in the tissue, the enzyme which causes the oxidation of *l*(+)-glutamic acid to α-ketoglutaric acid is without effect on the *d*-isomer. In an aqueous extract of the same tissue, the specificity seems to be reversed, so that only *d*(−)-glutamic acid is oxidized. The fundamental cause of these peculiarities in behavior is obscure.



Metabolism of Arginine. Mammalian liver contains an enzyme, *arginase*, capable of hydrolyzing arginine with the production of urea and ornithine (Kossel and Dakin).[36] Inasmuch as this enzyme is absent from the liver of birds, Clementi [37] proposed the generalization that arginase is present in the livers of all animals which form urea as the end-product of nitrogen metabolism and absent in those in which uric acid is the end-product. This viewpoint has since found support in the work of Krebs and Henseleit on the origin of urea (see p. 411).

In mammals, arginase occurs principally in the liver, but the kidney contains a small amount. Contrarily, in birds, the kidney is the richest source, and none is present in the liver.[38] It is of interest that in elas-

[34] Many writers now use the nomenclature introduced by Fischer, *Ber.*, **41**, 893 (1908) and Wohl and Freudenberg, *Ber.*, **56**, 309 (1923), according to which amino acids are designated *l*- and *d*- on the basis of their spatial configuration and without regard to the direction of optical rotation. All naturally occurring amino acids are included in the *l*-series; the unnatural amino acids are described as *d*-amino acids. To indicate the actual direction of rotation (+) or (−) is used. To illustrate, the natural glutamic acid was described as *d*-glutamic acid on p. 88. Using the Fischer-Wohl-Freudenberg nomenclature, it is designated *l*(+)-glutamic acid. The unnatural isomer is therefore *d*(−)-glutamic acid.

[35] *Biochem. J.*, **30**, 665 (1936).

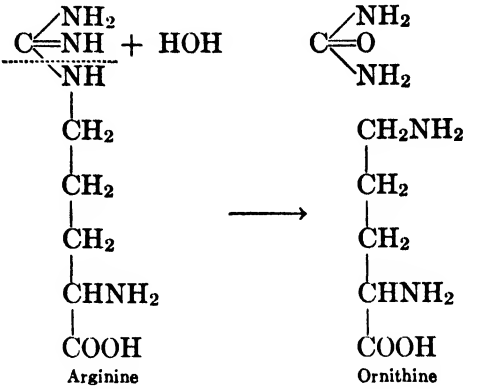
[36] *Z. physiol. Chem.*, **41**, 321 (1904); **42**, 181 (1904).

[37] A. Clementi, *Atti R. Accad. Lincei, Rendic.*, Series 5, **23**, 612 (1914); **27**, 299 (1918).

[38] A. Hunter and J. A. Dauphinee, *Proc. Roy. Soc.* (*London*), *B*, **97**, 227 (1925).

mobranchs, of which the dogfish is an example, arginase is abundant in all tissues.[39]

Arginase catalyzes the hydrolysis of arginine to ornithine and urea:



That this is necessarily the first step in the intermediary metabolism of arginine is not known. It is believed that ornithine is converted to succinic acid and eventually oxidized, but what the intermediate products are has not been determined. One of these intermediates, perhaps succinic acid, is capable of forming glucose, since both ornithine and arginine yield sugar in phlorhizin diabetes, in amounts sufficient to account for three carbon atoms, or one-half the total number in arginine (Dakin).[40]

Another pathway of metabolism has been suggested, namely one involving the conversion of arginine into guanidinebutyric acid, $(NH_2 \cdot \overset{\overset{NH}{\|}}{C} \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH)$, which by β-oxidation would yield guanidineacetic acid $(NH_2 \cdot \overset{\overset{NH}{\|}}{C} \cdot NH \cdot CH_2 \cdot COOH)$. By methylation, the latter would yield creatine $(NH_2 \cdot \overset{\overset{NH}{\|}}{C} \cdot NCH_3 \cdot CH_2 \cdot COOH)$. There is, however, no experimental evidence in support of this idea.[41] Guanidineacetic, guanidoacetic and guanidinoacetic are used synonymously. The conversion of guanidineacetic acid into creatine seems to

[39] This is related to the extraordinary production of urea in elasmobranchs. The blood of marine elasmobranchs contains about 2 per cent of urea; this is of importance in these organisms in the regulation of osmotic pressure. See E. Baldwin, "An Introduction to Comparative Biochemistry," Macmillan, 1937.

[40] H. D. Dakin, "Oxidations and Reductions in the Animal Body," 1922 edition, p. 81.

[41] K. Thomas and M. G. H. Goerne, *Z. physiol. Chem.*, **92**, 163 (1914); **104**, 73 (1918–19).

be established,[42] but arginine itself is not known to be a precursor of either creatine or creatinine (p. 421).

Convincing evidence of the synthesis of arginine in the body has been obtained in Rose's laboratory.[43] However, it appears that the rate of synthesis is not sufficiently rapid to meet the demands of normal growth. Accordingly, Rose has classified arginine among the essential amino acids (p. 569).

Metabolism of Lysine. Since Dakin has shown that lysine yields neither glucose nor acetone bodies in phlorhizin diabetes, all pathways of metabolism that would give rise to either ketogenic or anti-ketogenic substances must be excluded. Corley [44] states that ε-aminocaproic acid is also not a sugar-former in the completely phlorhizinized dog.

Lysine is not synthesized in the body and must therefore be provided in the diet. Its metabolism in embryonic development has been considered by Calvery.[45]

Metabolism of Cystine. The first step in the normal metabolic degradation of cystine is believed to be its reduction to form two molecules of cysteine.[46] If this is correct, the next step is probably an oxidative deamination, the ammonia being presumably converted to urea. The sulfur is for the most part oxidized to sulfate and is eliminated in the urine mainly as inorganic sulfate. A smaller amount is excreted as ethereal sulfates, conjugated substances formed in the detoxication of absorbed products of intestinal putrefaction, such as phenol and indoxyl. In addition, the urine contains a certain amount of unoxidized sulfur. It is probable that most of the inorganic sulfate is derived from exogenous metabolism, for the amount varies with the total nitrogen and particularly with the urea elimination. The significance of these urinary constituents will be further considered in other connections.

The possibility must be considered of direct oxidation of cystine and even of cystine in peptide combination, without preliminary reduction or hydrolysis.[47]

[42] L. Baumann and H. M. Hines, *J. Biol. Chem.*, **31**, 549 (1917); M. Bodansky, *ibid.*, **112**, 615 (1935–1936); **115**, 641 (1936).

[43] W. E. Bunney and W. C. Rose, *J. Biol. Chem.*, **76**, 521 (1928); C. W. Scull and W. C. Rose, *ibid.*, **89**, 109 (1930).

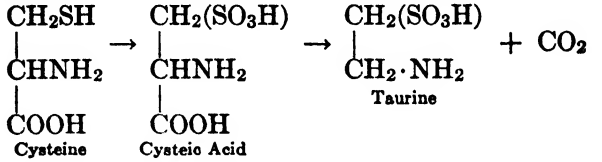
[44] *J. Biol. Chem.*, **81**, 545 (1929).

[45] *Ibid.*, **83**, 649 (1929); **95**, 297 (1932).

[46] On this point opinion is divided. According to Brand, Cahill, and Harris *J. Biol. Chem.*, **109**, 69 (1935), cystine can be catabolized without previous reduction, to cysteine, a conclusion based on the observations in a patient with cystinuria who was able to utilize cystine, but who excreted extra cystine when cysteine was fed. Opposed to Brand's view is the opinion of Pirie, *Biochem. J.*, **28**, 305 (1934), based on studies of the oxidation of cystine by slices of rat liver and kidney. Pirie's conclusion is that cystine can be oxidized only after undergoing reduction. In accord with this idea are the earlier experiments of Lewis and McGinty and Lewis, Updegraff and McGinty (*J. Biol. Chem.*, **53**, 349 [1922]; **59**, 59 [1924]), which showed that the administration of phenyluraminocystine and dibenzoylcystine results in the excretion of phenyluraminocysteine and dibenzylcysteine, respectively.

[47] A. White and J. B. Fishman, *J. Biol. Chem.*, **116**, 457 (1936).

Cystine is a constituent of glutathione (p. 303) and insulin (p. 465) and is the precursor of taurine. The last occurs in combination with cholic and choleic acids in taurocholic and taurocholeic acids, respectively. The formation of taurine from cysteine may be brought about by oxidizing the latter with bromine. The reactions are represented as follows:



It is possible, however, that in the body conjugation of the cholic acid occurs with cysteine and that the conjugated product is then oxidized to taurocholic acid. Taurine and cysteic acid are oxidized with difficulty, if at all (Schmidt and Clark),[48] though the latter is deaminized quite readily.

Cysteine is readily oxidized by iodine to cystine: $2R—SH + I_2 = R—S—S—R + 2HI$. The further action of iodine results in the slow oxidation of cystine to cysteic acid: $R—S—S—R + 5I_2 + 6H_2O = 2R—SO_3H + 10HI$.[49] Dissolved in hydrochloric acid, cystine is similarly oxidized by free oxygen, but much more slowly (Andrews).[50] However, in the presence of copper salts this reaction is greatly accelerated.

In unusual intoxications with foreign organic compounds, cystine may be used by the organism as the detoxicating agent. Thus the monohalogen derivatives of benzene are converted into the corresponding mercapturic acid derivatives and excreted [51] (p. 393).

Cysteine is quantitatively converted into glucose (carbon for carbon) in phlorhizin diabetes (Dakin),[52] probably with the intermediate formation of serine.

A large proportion of the cystine derived from the food is required for the synthesis of various types of keratins, found in hair, wool, feathers, and other epidermal structures. Wilson and Lewis,[53] found human hair to contain between 15.6 and 21.2 per cent of this amino acid.

Cystine accounts for practically all the sulfur in human hair, in wool, and in rabbit fur, but not in camel's hair.[54]

[48] *Ibid.*, **53**, 193 (1922).

[49] K. Shinohara, *ibid.*, **96**, 285 (1932).

[50] J. C. Andrews, *ibid.*, **97**, 657 (1932); **102**, 263 (1933).

[51] For further details and references to the literature consult C. P. Sherwin, "The Fate of Organic Compounds in the Animal Body," *Physiol. Rev.*, **2**, 264 (1922); A. M. Ambrose and C. P. Sherwin, "Detoxication Mechanisms," *Ann. Rev. Biochem.*, **2**, 377 (1933).

[52] *J. Biol. Chem.*, **14**, 321 (1913).

[53] *J. Biol. Chem.*, **73**, 543 (1927).

[54] C. Rimington, *Biochem. J.*, **23**, 726 (1929); **25**, 71 (1931); J. Barritt and Rimington, *ibid.*, **25**, 1072 (1931).

It appears, however, that the demands for protein (and cystine) for the growth of the body with its essential tissues and organs take precedence over the demands for the growth of hair. Lightbody and Lewis [55] have shown that, when diets of low cystine content are fed to rats, the growth of hair is much more markedly diminished than the general growth of the body. Similarly, the availability of cystine in the diet of sheep is apparently a factor which determines the cystine content of their wool.

Experiments conducted in Mitchell's laboratory [56] have shown that on a cystine-deficient diet the growth of hair in rats is small and the cystine content is lower than normal. Some of the hair also shows structural abnormalities, possessing a thinner cortex, which presumably represents the completely keratinized part of the hair. Martin and Gardner [57] have reported that cysteine, through the sulfhydryl group, acts as a stimulant to the hair follicle, bringing about a trichogenic action in hereditary hypotrichosis of the rat.

Many years ago it was shown that diets deficient in cystine are inadequate for growth (p. 563). This led to the obvious conclusion that cystine is not synthesized in the body. However, following the discovery of methionine, it was determined by Jackson and Block [58] that this amino acid was capable of replacing cystine in nutrition. Soon thereafter it was found that homocystine (p. 392), prepared from methionine, was also capable of replacing cystine.[59] These results signify the conversion of methionine to cystine, with homocysteine as the probable intermediate (p. 394). In a sense, therefore, cystine should not be classified among the indispensable amino acids, although it is probable that the total requirement is not always met by the methionine ordinarily available in the diet (see pp. 564, 570).

It should be pointed out that even such closely related compounds as taurine, cysteic acid, dithiodiglycollic acid,

$$(COOH \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot COOH)$$

β-dithiodipropionic acid

$$(COOH \cdot CH_2 \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CH_2 \cdot COOH)$$

and α-dihydroxy-β-dithiodipropionic acid

$$(COOH \cdot CHOH \cdot CH_2 \cdot S \cdot S \cdot CH_2 \cdot CHOH \cdot COOH)$$

are not changed to cystine or cysteine. Diets deficient in cystine and supplemented with these substances are inadequate for growth. How-

[55] *Ibid.*, **82**, 485 (1929).
[56] D. B. Smuts, H. H. Mitchell, and T. S. Hamilton, *J. Biol. Chem.*, **95**, 283 (1932).
[57] *J. Biol. Chem.*, **111**, 193 (1935).
[58] R. W. Jackson and R. J. Block, *ibid.*, **98**, 465 (1932); see also B. W. Chase and H. B. Lewis, *ibid.*, **101**, 735 (1933).
[59] Du Vigneaud, H. M. Dyer, and J. Harmon, *ibid.*, **101**, 719 (1933).

ever, dithiodiglycollic acid, β-dithiodipropionic acid, and α-dihydroxy-β-dithiodipropionic acid, administered orally or subcutaneously to rabbits, are all readily oxidized (Westerman and Rose).[60]

Cystinuria. Cystinuria is an abnormal condition in which cystine is present in the urine, apparently because of some failure in its metabolism. It seems to be hereditary and is said to occur somewhat oftener in males than in females. Garrod [61] has described cystinuria as an inborn error of metabolism.

Robson [62] has investigated the genealogical tree of a patient with pronounced cystinuria. There was a definite family history of this abnormality as shown by the accompanying diagram. It is to be regretted that no information was available concerning many members of this family. These are indicated by crosses. Despite these gaps, however, this family tree illustrates very clearly the hereditary nature of the disease.

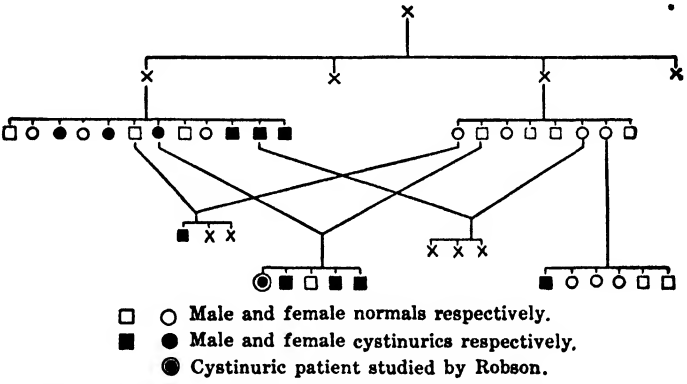


FIG 32.—Showing the family history of a patient with cystinuria. (Courtesy of Dr. Robson)

Cystinurics continue to excrete cystine even on a protein-free diet and during starvation, which would indicate that the cystine is at least partly endogenous in origin. The nature of the metabolic derangement is somewhat obscure. It has been repeatedly demonstrated that the cystinuric can utilize almost completely considerable amounts of the free amino acid.

Brand and associates [63] observed that, whereas cystine and glutathi-

[60] *Ibid.*, **79**, 423 (1928).

[61] Sir Archibald E. Garrod, "Inborn Errors of Metabolism," Oxford University Press, London, 2d edition, 1923.

[62] *Biochem. J.*, **23**, 138 (1929); see also E. M. Hickmans and W. C. Smallwood, *ibid.*, **29**, 357 (1935).

[63] E. Brand, G. F. Cahill, and H. M. Harris, *J. Biol. Chem.*, **109**, 69 (1935); Brand, Cahill and R. J. Block, *ibid.*, **110**, 399 (1935); compare with the negative results obtained with methionine by J. C. Andrews and A. Randall, *J. Clin. Investigation*, **14**, 517 (1935).

one are almost completely utilized in cystinuria, cysteine and methionine are excreted in a large measure as extra cystine. They believe that in the cystinuric the cystine is derived from dietary methionine and that the fundamental, inborn error is concerned with the handling of cysteine. Brand's work has been confirmed by Lewis, Brown, and White,[64] who have shown, moreover, that a high-protein diet favors the utilization of exogenous methionine and presumably also of the usual precursor (methionine?). This is a departure from the belief formerly held that cystinuria is augmented on a high-protein diet, either because of an increased exogenous supply of cystine, or because of stimulation by the protein of some processes of endogenous metabolism, resulting in the production of cystine

In a case of cystinuria studied by Brand and Biloon,[65] the freshly voided urine did not contain free cystine but a cystine complex of undetermined constitution. When the urine was allowed to stand this compound gradually decomposed, liberating the free amino acid.

That the finding of cystine crystals in the urine is too rigid a criterion for the detection of cystinuria is shown by Lewis's [66] survey of about 11,000 urine specimens obtained from healthy young men and women. Although only four individuals were found to excrete cystine crystals fourteen others gave a strongly positive reaction for this amino acid.

The occurrence in the urine of cystinurics of other amino acids, such as lysine and tyrosine, and the diamines, putrescine and cadaverine, has been reported on several occasions. In 1911, Ackermann and Kutscher [67] isolated lysine from the urine of a cystinuric patient. The same patient was in 1927 under the observation of F. A. Hoppe-Seyler,[68] who isolated arginine. Results of this nature are suggestive of a more generalized metabolic disturbance than one involving cystine alone. However, in the case studied by Robson, there was no evidence of the presence in the urine of lysine, tyrosine, putrescine, or cadaverine.

Owing to its insolubility, the cystine may contribute to the formation of urinary concretions.[69] It is usually stated that there are no other well-defined pathological symptoms, and, indeed, cystinurics have been known to live to a ripe old age.[70] On the contrary, Robson, in referring

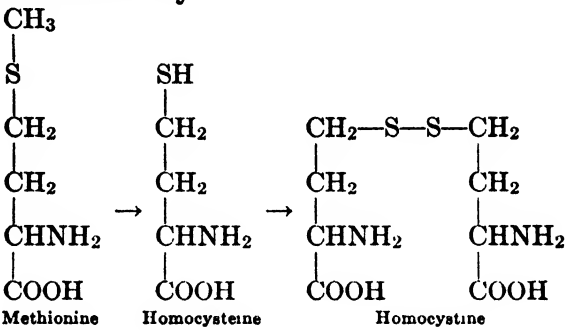
[64] *J. Biol. Chem.*, **114**, 171 (1936).
[65] *J. Biol. Chem.*, **86**, 315 (1930).
[66] *Ann. Internal Med.*, **6**, 183 (1932).
[67] *Z. Biol.*, **57**, 354 (1911).
[68] *Deut. Arch. klin. Med.*, **154**, 97 (1927).
[69] Cystine calculi may attain a considerable size. Tennant (*J. Am. Med. Assoc.*, **80**, 305 [1923]) removed fourteen concretions, aggregating 73 grams in weight, from two kidneys and a ureter. One of these weighed 50 grams. Mörner has also described a cystine stone weighing 50.2 grams (cited by H. B. Lewis, *Yale J. Biol. Med.*, **4**, 437 [1932]).
[70] E. Meyer (*Deut. Arch. klin. Med.*, **172**, 207 [1931]) has observed a case of cystinuria in a woman 87 years of age. Kaufmann, in his "Pathology," translated by Stanley P. Reimann, Philadelphia, 1929, Vol. II, p. 1413, described a case of cystinuria in a 21-month-old boy which came to autopsy. There were chalky

to his patient's history, makes this statement: "Special interest was taken in the case because of the strong family history of the disease, several members having already died from the consequences of this disturbance."

There are several reports of the occurrence of cystinuria in dogs. The possibility of its hereditary nature is suggested by recent observations in Brand's laboratory.[71]

Cystine, in small amounts, is excreted by normal individuals. In a study of 50 normal subjects, Medes[72] found the variation in daily output within the range of 0.8 to 84 mg.

Metabolism of Methionine. Demethylation is the first step in the metabolism of methionine. The product, homocysteine, may be converted into homocystine, or it may be converted into cysteine and in turn to cystine, or it may follow other paths in anabolism and catabolism. Although very little is known of the reactions involved, it seems to be well established that methionine and cystine are very closely related metabolically.



deposits of cystine in various internal organs such as the kidneys, walls of the intestines, mesenteric nodes, liver, and particularly in the spleen. Abderhalden (*Z. physiol. Chem.*, **38**, 557 [1903]), to whom the tissues were sent for analysis, made a careful investigation of the dead child's relatives. There were two living brothers, one 14 months and the other 5½ years old. Both were cystinurics. Another brother had died at 17 months and a sister at the age of 9½ months under apparently similar circumstances. It was established that the father of these children and the paternal grandfather had cystinuria. Negative results were obtained in the case of the mother, although Abderhalden states that when the urine was treated with alkali and lead acetate and heated a blackening was obtained. He was not able to determine the nature of the sulfur compound giving this test. The paternal grandmother was negative.

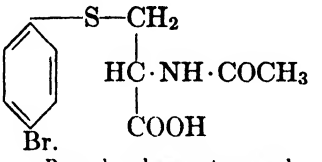
Equally interesting are the two cases of cystinuria described by Lignac (*Deut. Arch. klin. Med.*, **145**, 139 (1924), one in a 3-year-old boy and the other in a boy 2 years old. Both children were markedly underweight, had never learned to walk, and the older had stopped growing at the age of 2 years. On *post mortem* examination extensive deposits of cystine were found in all parts of the body, but especially in the kidneys. That cystinuria, particularly in the young, may be associated with a very severe and extensive pathology is clearly shown by these cases.

[71] *J. Biol. Chem.*, **114**, 91 (1936).

[72] *Biochem. J.*, **31**, 12 (1937).

The close relationship is partly brought out by the fact that albino rats kept on a cystine-deficient diet will resume growth when the deficiency is compensated by the addition to the diet of either *l*-cystine or methionine. It is an important fact that only *l*-cystine is utilized for the purpose and that *d*-cystine has no effect. On the other hand, *d*- and *l*-methionine are both utilized and replace *l*-cystine equally well. It has also been shown that *d*- and *l*-homocystine are equally well utilized for growth purposes by rats on a cystine-deficient diet. Dyer and du Vigneaud [73] consider this important evidence of the interrelationship in metabolism of homocystine and methionine.

Other evidence has been obtained through a study of the detoxication of monobrombenzene. This compound is ordinarily converted in the organism into *p*-bromphenylmercapturic acid, in which form it is excreted in the urine.[74]



p-Bromphenylmercapturic acid

In animals deprived of sulfur in their diet, a considerable proportion of the brombenzene, after oxidation to bromphenol, is conjugated with sulfuric acid and excreted as ethereal sulfate. However, when to such a deficient diet protein or *l*-cystine is added, *p*-bromphenylmercapturic acid is formed. Precisely the same result is obtained when the diet is supplemented with methionine, but not when taurine, for example, is fed.[75]

Additional information of the metabolism of methionine has been obtained in studies of the partition of the various sulfur fractions excreted in the urine. Of interest in this connection are the results of Stekol [76] showing that, in dogs maintained on a low cystine diet, *l*-cystine when fed was retained, whereas *dl*-cystine was only partly retained, a considerable part appearing in the urine. Under similar conditions *l*- and *dl*-methionine were almost completely and equally well retained.

On the basis of their observations in cystinuria and of other data, Brand, Cahill, and Block [63] have represented the catabolism of the sulfur amino acids by the following scheme.

[73] *J. Biol. Chem.*, **109**, 477 (1935).

[74] The reaction involves first oxidation to *p*-bromphenol and its subsequent conjugation with cysteine. The resulting *p*-bromphenylcysteine then undergoes acetylation, as indicated. Acetylation appears to be a fairly common reaction in the animal body.

[75] For details the student is referred to the papers of A. White and H. B. Lewis, *J. Biol. Chem.*, **98**, 607 (1932), and J. A. Stekol, *ibid.*, **117**, 147; **118**, 155 (1937). Earlier studies of detoxication through the formation of mercapturic acid have been reviewed by A. M. Ambrose and C. P. Sherwin, *Ann. Rev. Biochem.*, **2**, 377 (1933).

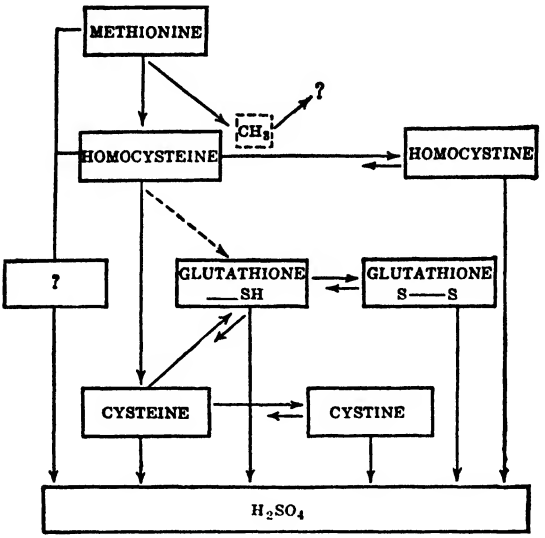


FIG. 33.—Oxidative catabolism of the sulfur amino acids.
(After Brand, Cahill and Block.)

Metabolism of Phenylalanine and Tyrosine. The first step in the metabolism of phenylalanine is believed to be its conversion into tyrosine (Embden and Baldes [77]). That a certain amount may escape this change has been shown by H. B. Lewis and associates.[78] Rabbits injected subcutaneously with phenylalanine were found to excrete considerable amounts of phenylpyruvic acid. The same result was obtained when phenylpyruvic acid itself was injected, indicating that this compound is not easily oxidized in the body. There was no evidence of excretion of *p*-hydroxyphenylpyruvic acid. In a similar study in which phenylalanine was administered *orally*, Kotake found the urine to contain both acids.

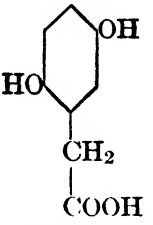
From these and other observations to be considered presently, it is probable that the main path of phenylalanine metabolism, after the initial stage of its conversion, is the path of tyrosine metabolism. Another path, which at present is considered to be of secondary importance, is independent of tyrosine and involves the formation of phenylpyruvic acid as an intermediate. Because the fate of the two amino acids is so largely the same, they will be studied together.

[76] *J. Biol. Chem.*, **109**, 147 (1935).

[77] *Biochem. Z.*, **55**, 301 (1913).

[78] N. F. Shambaugh, H. B. Lewis, and D. Tourtellotte, *J. Biol. Chem.*, **92**, 499 (1931); J. P. Chandler and H. B. Lewis, *ibid.*, **96**, 619 (1932). However, this compound was not recovered in the urine even after the administration of tyrosine, from which it may be concluded that, if *p*-hydroxyphenylpyruvic acid was formed, it readily underwent further metabolic change. Compare with Kotake, Masai, and Mori, *Z. physiol. Chem.*, **122**, 195 (1922).

Alcaptonuria. Before investigating the reactions involved in the metabolism of phenylalanine and tyrosine it is permissible to digress at this point in order to refer briefly to a peculiar and rare condition, called *alcaptonuria,* which appears to be hereditary, and in which there is obviously a derangement in the metabolism of these amino acids. Alcaptonuria occurs more frequently in males than in females. The urine of alcaptonurics, when allowed to stand exposed to the air, absorbs oxygen and turns black, owing to the presence of homogentisic acid,[79] which has the following formula:



Homogentisic acid

It is important to note that phenylalanine and tyrosine, when given to an alcaptonuric, are converted into homogentisic acid and excreted as such, but when given to a normal individual are oxidized completely. Moreover, when homogentisic acid itself is administered to a normal individual it is apparently oxidized, but in the alcaptonuric this is not the case. This signifies that homogentisic acid is probably an intermediate in the normal metabolism of phenylalanine and tyrosine, a view supported by the work of Embden, Salomon, and Schmidt,[77] who found that all three of these substances yield acetoacetic acid when perfused through a surviving liver.[80]

[79] Homogentisic acid was discovered by J. Marshall, *Am. J. Pharm.*, **59**, 131 (1887), and identified as 2,5-dihydroxyphenylacetic acid by M. Wolkow and E. Baumann, *Z. physiol. Chem.*, **15**, 228 (1891).

[80] Dakin, however, does not believe that homogentisic acid formation is a necessary step in the metabolism of tyrosine. According to this investigator, there is good evidence for believing that an immediate precursor of homogentisic acid is a compound having a quinonoid structure. Dakin (*J. Biol. Chem.*, **9**, 151 [1911]) therefore administered *p*-methylphenylalanine and *p*-methoxyphenylalanine,

$$(CH_3 \cdot C_6H_4 \cdot CH_2 \cdot CHNH_2 \cdot COOH)$$

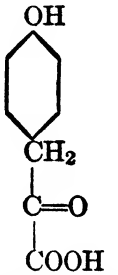
and

$$(CH_3O \cdot C_6H_4 \cdot CH_2 \cdot CHNH_2 \cdot COOH),$$

to alcaptonurics and showed that these substances were completely oxidized, presumably because of their inability to form quinonoid derivatives. Fromherz and Hermanns (*Z. physiol. Chem.*, **91**, 194 [1914]) performed similar experiments with *m*-methyltyrosine and *p*- and *m*-methylphenylalanine. These substances do not undergo the quinonoid transformation and, hence, did not give rise to homogentisic acid in these experiments. Accordingly, Dakin has postulated that alcaptonuria represents a condition in which there is not only an abnormal formation of homogentisic acid but also an abnormal failure to catabolize it when formed.

Alcaptonuric individuals who reach middle life may develop *ochronosis*, a condition in which the cartilages acquire a black pigmentation. Ochronosis has also been observed in persons who over a long period of years have applied carbolic acid dressings to ulcers of the legs. A detailed account of the chemical and clinical aspects of ochronosis is given by Sir Archibald E. Garrod in his monograph, "Inborn Errors of Metabolism." [81]

Tyrosinosis. A second abnormality in tyrosine metabolism has been described by Medes [82] under the term "tyrosinosis." Although only one case of this remarkable condition has been described thus far, its careful study by this investigator has given considerable information concerning several stages of the intermediary metabolism of tyrosine. Briefly stated, the condition described consists in a slowing up of the first steps of tyrosine metabolism and a complete stop at the stage of *p*-hydroxyphenylpyruvic acid. This was shown by the daily excretion from endogenous sources of about 1.6 grams of *p*-hydroxyphenylpyruvic acid (enol form).



p-Hydroxyphenylpyruvic acid

Increasing the protein intake, or feeding tyrosine, resulted in an increased elimination of this compound, but tyrosine and *l*-*p*-hydroxyphenyllactic acid were likewise excreted. When the ingestion of tyrosine was increased still further, the urine also contained *l*-3 : 4-dihydroxyphenylalanine. The last two compounds Medes considers to be products of side reactions and not in the main path of metabolism.

Of particular interest from the standpoint of normal metabolism is the fact that when phenylalanine was fed it caused an increased excretion of tyrosine and of *p*-hydroxyphenylpyruvic acid. When the lat-

[81] For clinical studies of alcaptonuria the student is referred to the following recent papers: H. Reinwein, *Deut. Arch. klin. Med.*, **170**, 327 (1931); P. Sachs, *ibid.*, **170**, 344 (1931); K. Ballowitz, *Jahrb. Kinderheilk.*, **134**, 182 (1932).

Ochronosis in cattle has been described by H. Fink, *Z. physiol. Chem.*, **197**, 193 (1931). The occurrence of alcaptonuria in a rabbit has been reported by J. Lewis, *J. Biol. Chem.*, **70**, 659 (1926).

Homogentisic acid has been detected in the serum and milk of a 26-year-old mother with alcaptonuria (F. Lanyar and H. Lieb, *Z. physiol. Chem.*, **203**, 135 (1931).

[82] *Biochem. J.*, **26**, 917 (1932).

ter compound was administered, it was excreted in the urine partly unchanged and partly as *l*-*p*-hydroxyphenyllactic acid. The last compound, when fed, was excreted unchanged.

According to Medes, these observations prove that the formation of *p*-hydroxyphenylpyruvic acid is an early step in the normal metabolism of tyrosine. In the case under consideration there was obviously a failure in the transformation of the *p*-hydroxyphenylpyruvic acid into 2 : 5-dihydroxyphenylpyruvic acid, as well as failure in the conversion of tyrosine into 2 : 5-dihydroxyphenylalanine. Especially striking was the observation that homogentisic acid, when fed, was completely oxidized. This strongly suggests that the 2 : 5 oxidation of tyrosine and of *p*-hydroxyphenylpyruvic acid, which are steps in the normal metabolism of tyrosine, was difficult for the patient with tyrosinosis, whereas a later step, namely the oxidation of homogentisic acid, is difficult for the alcaptonuric.

Phenylketonuria. Fölling [83] made the curious discovery of the occurrence of phenylpyruvic acid in the urine of certain mentally deficient individuals, a finding confirmed by Penrose.[84] The condition is familial and is inherited as a single Mendelian recessive character, resembling in this respect two other abnormalities of tyrosine and phenylalanine metabolism, namely alcaptonuria and albinism.[85] However, it is not so rare as either of these conditions. The abnormality has been described by Fölling as *imbecillitas phenylpyruvica;* Penrose and Quastel [86] have called it *phenylketonuria.* The latter workers found that the administration of *d*-, *l*-, or *dl*-phenylalanine produced an increased excretion of phenylpyruvic acid in phenylketonurics. In normal subjects *l*-phenylalanine was completely utilized, whereas the ingestion of either the *d*- or *dl*- variety produced a slight phenylketonuria. A slight increase in ketonic acids in the urine was observed in phenylketonurics after the administration of tyrosine, but on the whole this amino acid was metabolized normally.

Penrose and Quastel have suggested that the metabolic disturbance in phenylketonurics is due largely to a diminished rate of oxidation, or rupture, of the benzene ring in phenylpyruvic acid. The occurrence of this anomaly therefore adds to the evidence that besides metabolism *via* tyrosine there is at least one other metabolic path for phenylalanine.

Cell suspensions of liver oxidize tyrosine much more readily than phenylalanine, whereas similar preparations of kidney oxidize phenylalanine readily and tyrosine slowly, or not at all (Bernheim and Bernheim).[87]

[83] *Z. physiol. Chem.*, **227**, 169 (1934).

[84] *Lancet*, **1**, 23; **2**, 192 (1935).

[85] It has not been established that tyrosinosis is also an hereditary condition, although this is suspected.

[86] *Biochem. J.*, **31**, 266 (1937).

[87] *J. Biol. Chem.*, **107**, 275 (1934); see also H. Krebs, *Z. physiol. Chem.*, **217**, 191 (1933).

From this it has been surmised that the phenylalanine → phenylpyruvic acid, etc., reaction may be the preferential path of breakdown in kidney (Edson [88]).

Intermediates of Tyrosine and Phenylalanine Metabolism. We may now inquire whether the metabolic products described in the preceding paragraphs may be made to fit into a logical scheme that would provide an insight into the sequence of reactions involved in the metabolism of tyrosine and phenylalanine.

To recapitulate some of the known facts:

(1) The conversion of *dl*-phenylalanine into *l*-tyrosine was demonstrated by Embden in liver perfusion experiments. He also found that both these amino acids yielded acetoacetic acid.

The formation of ketones from *d*- and *l*-phenylalanine and *l*- and *dl*-tyrosine has been confirmed by Edson,[88] using the liver slice method. In fact, it was shown that these are among the most strongly ketogenic amino acids.

(2) *p*-Hydroxyphenylpyruvic acid, which has been considered as an intermediate, also forms acetoacetic acid in perfused liver and liver slices. On the other hand, phenylpyruvic acid does not give rise to acetoacetic acid in liver.

(3) Homogentisic acid also forms large amounts of acetoacetic acid in perfused liver and liver slices.[89]

(4) The administration of tyrosine or phenylalanine causes an increased output of homogentisic acid in the alacaptonuric.

Neubauer's Theory of Tyrosine Metabolism. The foregoing considerations are in the main consistent with Neubauer's [90] theory of the intermediary metabolism of tyrosine, according to which two paths of oxidation are open, one leading through *p*-hydroxyphenylpyruvic acid, and the other through 2 : 5 dihydroxyphenylalanine. Both lead to 2 : 5 dihydroxyphenylpyruvic acid and in turn to homogentisic acid. The next step involves cleavage of the benzene ring with the formation of an open-chain compound.

These stages in the metabolism of tyrosine may be represented as follows (p. 399):

[88] *Biochem. J.*, **29**, 2498 (1935).

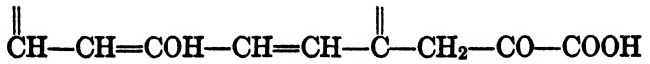
[89] Embden found that phenyllactic acid, $C_6H_5 \cdot CH_2 \cdot CHOH \cdot COOH$, produced acetoacetic acid. This is contrary to the result obtained by Edson.

There seem to be no data on the effect of feeding *p*-hydroxyphenylpyruvic acid, although it is very probably a precursor of homogentisic acid. The observation of Neubauer and Falta, *Z. physiol. Chem.*, **42**, 81 (1904), that phenylpyruvic acid (and phenyllactic acid), like phenylalanine, causes an increase in homogentisic acid output in the alacaptonuric is difficult to reconcile with its failure to form acetoacetic acid. There is perhaps some basis for the conjecture that the metabolism of phenylpyruvic acid in the *kidney* may involve the introduction of hydroxyl groups into the benzene ring and the production in turn of homogentisic acid and acetoacetic acid as intermediates.

[90] O. Neubauer, "Intermediarer Eiweisstoffwechsel," in "Handbuch der normalen und pathologischen Physiologie," Berlin, p. 862.

Tyrosine
p-Hydroxyphenylpyruvic acid
2 : 5 Dihydroxyphenylalanine
2 : 5 Dihydroxyphenylpyruvic acid
2 : 5 Dihydroxyphenylacetic acid (Homogentisic acid)
Opening of ring
Acetoacetic acid
$CO_2 + H_2O$

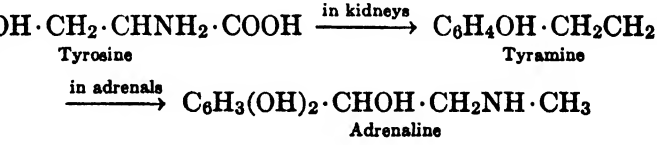
Opposed to Neubauer's theory is Dakin's conception,[91] according to which the path of oxidation of tyrosine lies only through *p*-hydroxyphenylpyruvic acid, in which compound opening of the ring occurs normally. This would yield the following compound:



Melanin. Tyrosine is oxidized by the enzyme tyrosinase to the brownish black pigment melanin which occurs normally as the coloring matter of hair, in the choroid of the eye, and in the skin, particularly in the dark races. In the hereditary condition described as albinism, there is apparently a failure in the formation of melanin. Pathologically melanin occurs in large amounts in melanotic tumors (usually melanosarcomas). If there is extensive development of such a tumor, melanin may occur in the urine (melanuria). Cases of melanuria have, however, been reported in which a melanotic tumor was not demonstrable. The analyses of melanin that are to be found in the literature would seem to indicate a variable composition, but this is probably due to the presence of other cell constituents in the materials analyzed.[92]

The formation of melanin from tyrosine has been studied by Raper,[93] who has succeeded in isolating several of the intermediary products.

Adrenaline and Thyroxine. Tyrosine is the mother substance of adrenaline (epinephrine, also suprarenine), the hormone of the adrenal (suprarenal) medulla. Recent studies by means of the surviving tissue slice method suggest that the first step is decarboxylation to tyramine, a reaction which occurs in the kidney.[94] The final stage in the conversion occurs in the adrenals.



Another very important product, derived from tyrosine (and phenylalanine), is thyroxine, the hormone of the thyroid gland (p. 474).

Synthesis. It has long been the prevailing opinion that tyrosine and phenylalanine were interchangeable in metabolism and that therefore

[91] H. D. Dakin, "Oxidations and Reductions in the Animal Body," pp. 84, et seq.

[92] For an excellent review of the subject, the student is referred to H. G. Wells, "Chemical Pathology," 5th edition, Saunders, Philadelphia (1925), Chapter XX; see also H. Waelsch, "Zur Kentniss der natürlichen Melanine," *Z. physiol. Chem.*, **213**, 35 (1932). For a summary of recent work consult S. Edlbacher, *Ann. Rev. Biochem.*, **6**, 269 (1937).

[93] H. S. Raper, *Biochem. J.*, **20**, 735 (1926), **21**, 89 (1927); *Physiol. Rev.*, **8**, 245 (1928); W. L. Duliere and Raper, *Biochem. J.*, **24**, 239 (1930).

[94] W. Schuler, H. Bernhardt, and W. Reindel, *Z. physiol. Chem.*, **243**, 90 (1936); Schuler and A. Wiedemann, *ibid.*, **233**, 235 (1935).

the deficiency of one in the diet would be compensated for by the presence of a sufficient amount of the other. But this viewpoint has changed radically since Womack and Rose [95] conclusively demonstrated that tyrosine is totally incapable of replacing phenylalanine for growth purposes (p. 569). The position of the latter as an indispensable amino acid is therefore established. On the other hand, the evidence points to the fact that diets otherwise adequate, but devoid of tyrosine, will support growth. Considering the ability of phenylalanine to form tyrosine, a deficiency of the latter in the diet would be corrected by the presence of phenylalanine. This is predicated on the assumptions that a sufficient supply of phenylalanine is available and that it is easily converted into tyrosine.

Tyrosinuria. In severe liver disease tyrosine crystals are often present in the urine. This is usually explained on the basis of disturbed hepatic function and diminished ability to deaminize amino acids. Tyrosine being relatively insoluble separates out. Recent quantitative studies [96] of tyrosine excretion in various diseases make it appear that, although this factor is significant, the principal cause of tyrosinosis is destruction or autolysis of liver tissue, such as occur, for example, in acute yellow atrophy.

Metabolism of Histidine. The fate of histidine in metabolism is obscure, and recent investigations have elucidated the problem only slightly. It is not a sugar-forming amino acid (Rapport),[97] nor is it known to give rise to acetone bodies.[98]

Leiter [99] found that histidine injected intravenously into dogs was almost completely utilized. Even when as much as 5 grams were given, the increase in urinary imidazoles could account for only about 150 mg. of histidine. On the contrary, the injection of 1-gram quantities of methylimidazole and methylimidazolelactic acid resulted in an excretion in the first 24 hours of approximately 30 and 40 per cent, respectively. Imidazole itself was not utilized appreciably, for 93 per cent of the 0.5 gram of this substance injected was recovered in the urine. Leiter states that in every case the increased urinary imidazole output was due entirely to the presence of the same imidazole as the one injected. These results indicate that the body has a high capacity for destroying the imidazole ring when attached to a side chain, particularly as in histidine, but that it does not have this capacity in the absence of the side chain. Leiter's conclusion was that none of the

[95] *J. Biol. Chem.*, **107**, 449 (1934); see also R. S. Alcock, *Biochem. J.*, **28**, 1174 (1934).

[96] S. S. Lichtman, *Arch. Internal Med.*, **53**, 680 (1934); see also H. G. Wells and P. Bassoe, *J. Am. Med. Assoc.*, **44**, 685 (1904).

[97] D. Rapport, *Physiol. Rev.*, **10**, 349, and especially 397 (1930).

[98] Compare H. D. Dakin and A. J. Wakeman, *J. Biol. Chem.*, **10**, 499 (1912); Dakin, *ibid.*, **14**, 321 (1913); M. Konishi, *Z. physiol. Chem.*, **122**, 237 (1922); N. L. Edson, *Biochem. J.*, **29**, 2498 (1935).

[99] *J. Biol. Chem.*, **64**, 125 (1925).

compounds which he used was an intermediary in the metabolism of any of the others. In evaluating Leiter's data in the light of later investigations, the possibility of partial conversion of imidazolelactic acid to histidine, in his experiments, should be considered.

According to Edlbacher,[100] the first step in the metabolism of histidine is disruption of the imidazole ring through the action of a specific enzyme, *histidase*, which occurs in the liver.

Histidinuria. Attention may be directed at this point to the presence of abnormally large amounts of histidine in the urine of gravid women. It has been reported that the excretion of histidine begins in the fifth week of pregnancy and continues until about the third day post-partum (Kapeller-Adler [101]). These findings have been correlated with the disappearance of active histidase from the liver of gravid women. The inference is that, in the absence of this enzyme, a considerable part of the histidine escapes metabolism. It has been stated further that this assures to the fetus an adequate supply of histidine. These ideas are of much interest, but it should be realized that on the basis of present knowledge all explanations of the significance of histidinuria must be regarded as unproved and hence tentative.

Histamine. The formation of histamine from histidine in the alimentary tract has been described (p. 210). Decarboxylation has been attributed to the action of certain bacteria, especially those of the colon-typhoid group. It is often stated that the histamine occurring in the various organs of the body (lung, liver, muscle, kidney, gastric and intestinal mucosa, etc.) has its origin in the alimentary canal, from which it is absorbed. This seems unlikely, for the probability is that at least in certain tissues decarboxylation of histidine may occur. Evidence for this reaction in the kidney has been recently submitted by Werle.[102]

Most tissues contain an histamine-decomposing enzyme, histaminase. The notable exception is lung, which does not contain this enzyme.[103]

[100] Compare: S. Edlbacher and J. Kraus, *Z. physiol. Chem.*, **191**, 225 (1930); **195**, 267 (1931); Edlbacher and M. Neber, *ibid.*, **224**, 261 (1934); E. Abderhalden, *ibid.* **200**, 87 (1931); F. Kauffmann and E. Mislowitzer, *Biochem. Z.*, **226**, 325 (1930).

Koessler and Hanke (*J. Biol. Chem.*, **59**, 803 [1924]), estimated the daily urinary excretion of imidazole complexes in normal individuals to vary between 120 and 220 mg. Considerably higher values (150 to 600 mg.) have been reported by Kauffmann and Engel (*Z. klin. Med.*, **114**, 405 [1930]). The lowest concentrations have been encountered in nephritis; relatively high values have been obtained in diseases of the liver.

It has been suggested that urocanic acid, β-imidazoleacrylic acid, may be an intermediate product of the metabolism of histidine. Its occurrence in dogs' urine, especially after feeding large amounts of histidine, and in the urine of the coyote has been reported. Others have failed to find this constituent in the urine and consider it a by-product, rather than an important intermediate of histidine metabolism.

[101] *Klin. Wochschr.*, **13**, 1220 (1934); **14**, 1790 (1935); *Biochem. Z.*, **264**, 131 (1933); **280**, 232 (1935).

[102] *Biochem. Z.*, **288**, 293 (1936).

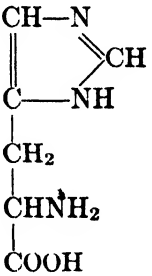
[103] C. H. Best and E. W. McHenry, *J. Physiol.*, **70**, 349 (1930).

This doubtless accounts for the fact that histamine accumulates in this organ; indeed lung tissue has come to be regarded as the body's chief reservoir for this substance. An increase in the histamine content of the lungs has been observed in guinea pigs, after the administration of histidine.[104]

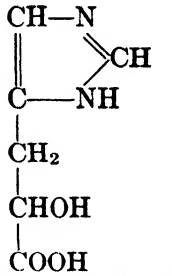
Histamine is of physiological importance largely because of its vasodilator effect and its action as a stimulant of gastric secretion (see also p. 172).

Synthesis. The inability of the organism to synthesize histidine and hence its indispensability in nutrition seems to be well established. It has been shown, however, that both in metabolism and for growth histidine may be replaced by imidazolelactic acid.[105] Carnosine (p. 632) can also support the growth of the white rat on a histidine-deficient diet.[106]

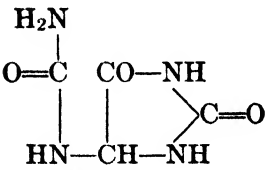
Purine Precursor. Histidine is one of the sources of purines in metabolism. Rats maintained on an histidine-free diet excrete less uric acid and allantoin than normally. With the addition of histidine to the deficient diet, there is an increased excretion of these constituents, as well as of creatinine (Rose and Cook).[107] The following formulas bring out the structural relationship between histidine, imidazolelactic acid, allantoin, and uric acid:



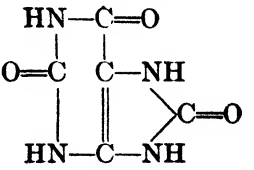
Histidine



Imidazolelactic acid



Allantoin



Uric acid

Ergothioneine. In 1909, Tanret [108] isolated a base from ergot, which

[104] W. Bloch and H. Pinösch, *Z. physiol. Chem.*, **239**, 236 (1936).

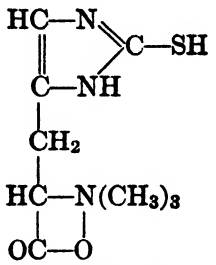
[105] G. J. Cox and W. C. Rose, *J. Biol. Chem.*, **68**, 781 (1926); see also B. Harrow and C. P. Sherwin, *ibid.*, **70**, 683 (1926).

[106] V. du Vigneaud, R. H. Sifferd, and G. W. Irving, *J. Biol. Chem.*, **117**, 589 (1937).

[107] *Ibid.*, **64**, 325 (1925).

[108] *J. pharm. chim.*, **30**, series 6, 145 (1909).

Barger and Ewins [109] identified as the betaine of thiolhistidine, represented by the following structural formula:



Ergothioneine
(Thioneine)

This substance was named ergothioneine. Several years ago it became apparent that the blood corpuscles contained a hitherto unknown sulfur compound. This was eventually shown to be identical with ergothioneine.[110, 111] Benedict and his associates have suggested that the term ergothioneine be contracted to *thioneine*. Human blood is reported to have 10–25 mg. per 100 cc. of this substance, and hog's blood, 14.5 mg. per 100 cc. However, considerable variations have been observed in the blood of pigs from different localities. Eagles and Vars [112] have reported certain experiments on pigs which they had fed protein hydrolyzates and suggest that the precursor of thioneine may be thiolhistidine. The physiological significance of thioneine remains to be determined.

Muscle contains a compound *l*-carnosine, first isolated by Gulewitsch and Amiradzibi,[113] which has been shown to be β-alanylhistidine. It exerts a marked depressor effect on blood pressure. *d*-Carnosine, the enantiomorph of the naturally occurring form, is physiologically inactive (du Vigneaud and Hunt[114]). Closely related to carnosine is anserine, or β-alanylmethylhistidine, discovered in goose muscle and more recently in certain fishes by Ackermann and associates.[115] Its synthesis from *l*-1-methylhistidine has been described by Behrens and du Vigneaud.[116]

Metabolism of Proline and Hydroxyproline. The structural relationship of proline, hydroxyproline and glutamic acid, which is brought

[109] *Trans. Chem. Soc.*, **99**, 2336 (1911).

[110] E. B. Newton, S. R. Benedict, and H. D. Dakin, *Science*, **64**, 602 (1926); *J. Biol. Chem.*, **72**, 367 (1927).

[111] B. A. Eagles and T. B. Johnson, *J. Am. Chem. Soc.*, **49**, 575 (1927); Hunter and Eagles, *J. Biol. Chem.*, **72**, 123 (1927).

[112] *J. Biol. Chem.*, **80**, 615 (1928).

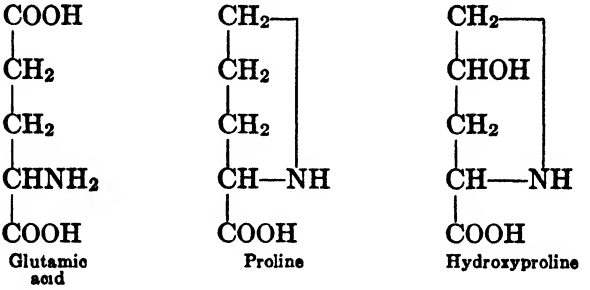
[113] *Ber.*, **33**, 1902 (1900); *Z. physiol. Chem.*, **30**, 565 (1900).

[114] *J. Biol. Chem.*, **115**, 93 (1936).

[115] D. Ackermann, O. Timpe, and K. Poller, *Z. physiol. Chem.*, **183**, 1 (1929); Ackermann and F. A. Hoppe-Seyler, *ibid.*, **197**, 135 (1931).

[116] *J. Biol. Chem.*, **120**, 517 (1937).

out by the following formulas, suggests that these compounds may share a common fate in metabolism.



Evidence in support of this idea has been submitted by Malherbe and Krebs.[117] After incubating kidney tissue with proline they were able to demonstrate the formation of α-ketoglutaric acid, ammonia, and what they suspected to be glutamine. A small amount of arsenious oxide was added to the substrate in order to check the oxidation of the amino acid at the stage of ketonic acid, thereby assuring its accumulation in sufficient amount for the purpose of isolation and identification. On the basis of their results, Malherbe and Krebs [117] believe that the metabolism of proline may be formulated as follows:

$$\text{Proline} + O_2 \rightarrow \text{glutamic acid} \begin{cases} + NH_3 \rightarrow \text{glutamine} \\ + \tfrac{1}{2}O_2 \rightarrow \alpha\text{-ketoglutaric acid} + NH_3 \end{cases}$$

The principal gap in this scheme is the transition from proline to glutamic acid. Pyrolidonecarboxylic acid,

$$(\overline{CO \cdot CH_2 \cdot CH_2 \cdot CHNH} \cdot COOH),$$

considered by others as a probable intermediate, is dismissed on the grounds that it is itself not oxidized under the conditions which favor the rapid metabolism of proline.

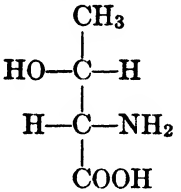
Hydroxyproline is oxidized by certain tissues under essentially the same conditions that favor the oxidation of proline. In the experiments of Malherbe and Krebs,[117] presumptive, but not definite, evidence was obtained of the formation of glutamine. The production of the latter would imply reduction of hydroxyproline to proline as the initial reaction in the metabolism of the hydroxyproline. Even if this reaction occurs, it seems from the work of Edson [88] that perhaps the greater part of hydroxyproline is metabolized by a different path. Edson [88] found that, whereas proline (rat liver slice method) does not form acetoacetic acid, hydroxyproline is markedly ketogenic, being excelled in this

[117] *Biochem. J.*, **29**, 2077 (1935).

respect by only three other amino acids, namely, leucine, tyrosine, and phenylalanine.

Like glutamic acid, proline is glucogenic. It was shown by Dakin that, in the completely phlorhizinized dog, the amount of extra glucose excreted after the administration of either proline or glutamic acid accounted for 3 of the 5 carbon atoms in the molecule.

Metabolism of α-Amino-β-Hydroxy-*n*-butyric Acid; *d*(—)-Threonine. The successful attempt at the isolation of this amino acid from protein and its identification grew out of the observation by Rose and associates [118] that young rats failed to maintain themselves on a diet in which the protein was replaced by a mixture of the twenty known amino acids, each of which was supplied in pure form. A search among the products of protein hydrolysis for the missing active principle, necessary to induce maximum growth, disclosed that it was α-amino-β-hydroxy-*n*-butyric acid. The naturally occurring amino acid is levorotatory, and its spatial configuration is analogous to that of *d*-threose (p. 47); hence its designation as *d*(−)-threonine, following the nomenclature of Fischer-Wohl-Freudenberg (p. 385).



d (−)-Threonine

The indispensability of this amino acid to proper nutrition seems to be definitely established.[119]

Judging by a report from Knoop's laboratory,[120] the metabolism of α-amino-β-hydroxybutyric acid differs from that of simple amino acids. Oxidation occurs at the β-carbon atom, as shown by the excretion of phenylacetic acid following the administration of γ-phenyl-β-hydroxy-α-aminobutyric acid. This is to be contrasted with the excretion of

[118] M. Womack and W. C. Rose, *J. Biol. Chem.*, **112**, 275 (1935); R. H. McCoy, C. E. Meyer, and W. C. Rose, *ibid.*, **112**, 283 (1935); C. E. Meyer and W. C. Rose, *ibid.*, **115**, 721 (1936). H. D. West and H. E. Carter, in Rose's laboratory, synthesized formyl-*dl*-O-methylthreonine, which they resolved by means of brucine. The optically active formyl derivatives were then converted into *d*(−) and *l* (+) threonine with 48 per cent hydrobromic acid (*J. Biol. Chem.*, **119**, 109 [1937]).

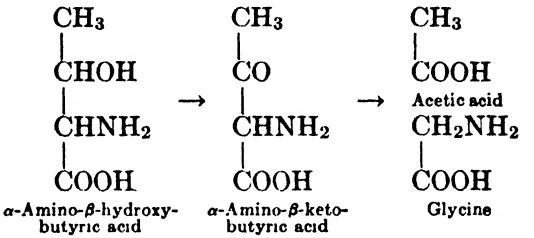
[119] W. C. Rose, *Harvey Lectures*, Series **30**, 49 (1934–5); *Science*, **86**, 298 (1937).

[120] *Z. physiol. Chem.*, **239**, 30 (1936).

Compare with the metabolism of fatty acids, p. 355. Further evidence that β-hydroxy-α-amino acids are oxidized at the β-carbon atom is given by the result with δ-phenyl-β-hydroxy-α-aminovaleric acid. This yields benzoic acid. Here, it is supposed, oxidation occurs at the β-carbon atom, as in the case of γ-phenyl-β-hydroxy-α-aminobutyric acid, and is followed by oxidation, again at the β-carbon atom.

benzoic acid after feeding the phenyl derivative of α-aminobutyric acid. Presumably this compound is oxidized first at the α-carbon atom, the product then undergoing β-oxidation.

The evidence presented by Knoop is obviously indirect. There is no certainty that the conclusions based on the reactions of the foreign phenyl derivative are entirely applicable to the amino acid itself. But if it is assumed, tentatively, that they behave alike in metabolism, the oxidation of the amino acid may be represented as follows:



From this it would appear that *d*(−)-threonine may be a potential source of glycine in metabolism.

Tryptophane Metabolism. From the urine of certain animals (dog, rabbit, rat, guinea pig, but not man or cat) [121] there has been isolated a compound, *kynurenic acid*, which is obviously a product of tryptophane metabolism. Several theories for its formation have been proposed, one being that of Ellinger and Matsuoka,[122] according to which the first step is the formation of indolepyruvic acid. When this substance is injected or fed to rabbits, it behaves like tryptophane in producing kynurenic acid. The various changes according to Ellinger's theory are indicated on page 408.

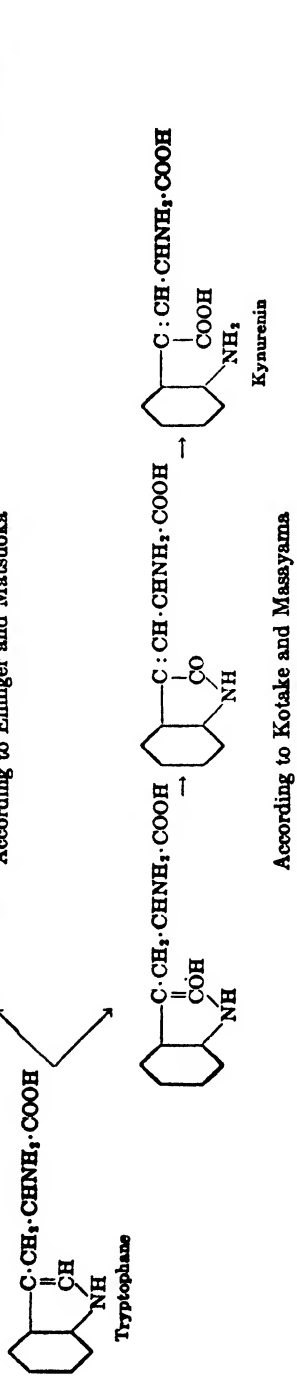
A second compound has been isolated from the urine of rabbits after they have been fed relatively large amounts of tryptophane. This has been identified as *kynurenin*. It has not been definitely established whether kynurenin is a precursor of kynurenic acid, or whether it is an intermediate in an alternative and distinct path of tryptophane metabolism.

Inasmuch as Ellinger and Matsuoka's theory makes no provision for the formation of kynurenin, Kotake [123] has proposed a somewhat different hypothesis. This, however, makes no allowance for the production of indolepyruvic acid.

[121] See W. G. Gordon, R. E. Kaufman, and R. W. Jackson, *J. Biol. Chem.*, **113**, 125 (1936), for references to original investigations.

[122] *Z. physiol. Chem.*, **109**, 259 (1920); Z. Matsuoka and Y. Yoshimatsu, *ibid.*, **143**, 206 (1925).

[123] Y. Kotake and associates, *ibid.*, **195**, 139 et seq. (1931); **214**, 1 (1933); **243**, 237 (1936); Kotake, *Ergeb. Physiol.*, **37**, 245 (1935).


Tryptophane
Indolepyruvic acid
According to Ellinger and Matsuoka
Aminobenzoylpyruvic acid
Kynurenic acid
According to Kotake and Masayama
Kynurenin

There is evidence that kynurenic acid is formed in the liver, and that, at least in dogs, a considerable proportion is excreted in the bile. The prevailing conception is that kynurenic acid is not a link in the chain of normal tryptophane oxidation in the animal body, but rather that it is an end-product of a set of side reactions brought into play especially when tryptophane is administered in excess of ordinary metabolic requirements (Jackson and Jackson).[124] In view of this it is somewhat difficult to explain the disappearance of ingested kynurenic acid in man.[125]

Life cannot be maintained indefinitely on a diet lacking tryptophane, for the body cannot synthesize this amino acid *de novo*. Tryptophane therefore belongs to the group of indispensable amino acids. However, it has been shown that indolepyruvic acid can serve as a biological substitute for tryptophane,[126] suggesting the reversibility of the tryptophane → indolepyruvic acid transformation. Tryptophane may also be replaced by certain of its amide derivatives (Baguess and Berg).[125] In general, compounds which like tryptophane support growth in the rat are capable of producing kynurenic acid in the rabbit, this animal being especially suitable for the experimental study of this aspect of tryptophane metabolism. *d*-Tryptophane seems to be an exception, for, although it supports growth as well as *l*-tryptophane (compare this with the behavior of *d*- and *l*-cystine), it produces no appreciable increase in the output of kynurenic acid. This suggests that there is a difference in the intermediary metabolism of the two enantiomorphs. Kynurenin is not utilized as a substitute for tryptophane.

It has been reported that tryptophane and kynurenin give rise to the urinary pigment urochrome.[127]

Kynurenic acid and kynurenin are further oxidized, but the steps involved are unknown. It is apparent that, although an enormous amount of work has been done in the attempt to trace the paths of tryptophane metabolism, the present state of knowledge is still far from satisfactory.

[124] *J. Biol. Chem.*, **96**, 697 (1932).

[125] The formation of kynurenic acid from various tryptophane derivatives has been investigated by C. P. Berg, *J. Biol. Chem.*, **91**, 513 (1931); **104**, 373 (1934); L. C. Bauguess and Berg, *ibid.*, **104**, 691 (1934); **106**, 615 (1934); **114**, 253 (1936).

[126] R. W. Jackson, *J. Biol. Chem.*, **84**, 1 (1929); C. P. Berg, W. C. Rose, and C. S. Marvel, *ibid.*, **85**, 219 (1929).

[127] Y. Kotake and H. Sakata, *Z. physiol. Chem.*, **195**, 184 (1931).

CHAPTER XIV

INTERMEDIARY METABOLISM OF PROTEIN (Continued)

UREA

In mammals, amphibia, and the elasmobranch fishes, the chief end-product of protein metabolism is urea; in birds and reptiles, it is uric acid (except in turtles, which excrete urea). In teleosts the end-product of nitrogen metabolism is ammonia.[1] The amount of urea formed depends on the diet, being greater on a high-protein diet than on a low one. A normal adult, on a protein intake of 100 to 120 grams, excretes about 30 grams of urea, which, expressed in terms of nitrogen, is equivalent to about 15 grams. The urea nitrogen excreted in twenty-four hours usually varies between 80 and 90 per cent of the total nitrogen, but when the total nitrogen is very low (4–7 grams), the percentage in the form of urea nitrogen is much lower (about 50 or 60 per cent of the total nitrogen).

A portion of the urea has its origin in arginine, but the major part is formed in the body from the ammonia which is split off in the deamination of amino acids. It has been supposed that the ammonia combines with carbon dioxide to form ammonium carbonate which, by the loss of one molecule of water, yields ammonium carbamate and, by the loss of a second molecule, yields urea. These reactions are indicated by the following formulas:

$$2NH_3 + CO_2 + H_2O \rightleftarrows \underset{\text{Ammonium carbonate}}{C(=O)(ONH_4)_2} \rightleftarrows \underset{\text{Ammonium carbamate}}{C(=O)(ONH_4)(NH_2)} + H_2O \rightleftarrows \underset{\text{Urea}}{C(=O)(NH_2)_2} + H_2O$$

The conception of Fearon [2] that cyanic acid is an intermediate in the formation of urea as well as in its decomposition by urease has been definitely disproved by Sumner and associates.[3] In the enzymic hydrolysis of urea, Sumner considers ammonium carbamate to be the intermediate product.

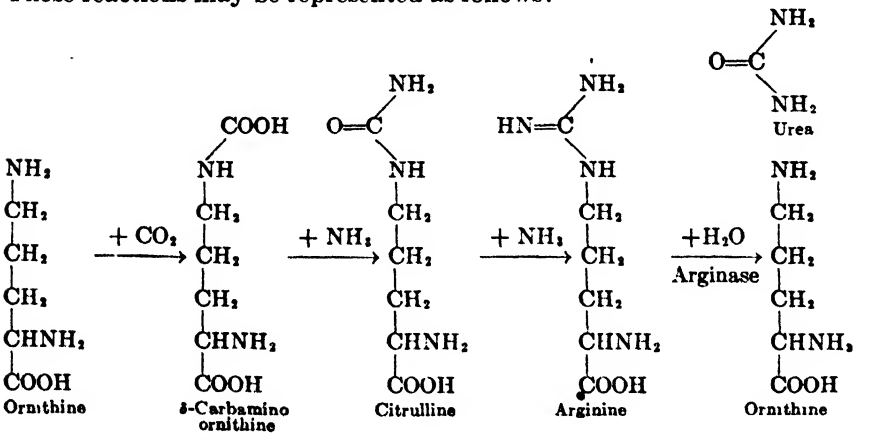
[1] E. Baldwin, "An Introduction to Comparative Biochemistry," Macmillan, 1937.

[2] *Biochem. J.*, **17**, 84, 800 (1917); *Physiol. Rev.*, **6**, 399 (1926).

[3] J. B. Summer, *J. Biol. Chem.*, **68**, 101 (1926); Sumner, D. B. Hand, and R. G. Holloway, *ibid.*, **91**, 333 (1931). See also N. N. Iwanoff, *Biochem. Z.*, **150**, 108 (1924); H. D. Kay, *Biochem. J.*, **17**, 277 (1923).

A new theory has been advanced by Krebs and Henseleit,[4] based on a large number of determinations of the utilization of ammonia and carbon dioxide and the production of urea by slices of various organs kept in appropriate nutritive solutions. Of all the tissues studied, only the liver produced urea under these conditions. The rate of its formation from ammonia and carbon dioxide was greatly accelerated by the addition of *ornithine*. In the presence of this substance, Krebs and Henseleit found the ratio of ammonia consumed to urea formed to be 1.81, as contrasted with the theoretical value of 2.0, indicating that some of the ammonia was derived from sources other than the added ammonia, presumably amino acids contained in the tissue. A large variety of amino acids and nitrogenous bases were tested, but none produced the acceleration characteristic of ornithine with the exception of *citrulline*, derived from ornithine by the addition of a molecule each of ammonia and carbon dioxide. In the presence of citrulline, the ratio 1.38 was obtained, which suggested it to be an intermediate—indeed, the first product in the conversion of ammonia into urea.

A more recent observation by Krebs [5] is that in the presence of phosphate buffer urea synthesis is almost completely depressed, but when a bicarbonate-CO_2 buffer is used the synthesis is greatly accelerated, depending on the concentration of buffer. The specific effect of the carbonic acid has been associated with the formation of δ-carbamino ornithine as the first step in the synthesis. Accordingly the formation of citrulline may now be considered as the second step. The third stage is a reaction between citrulline and a molecule of ammonia, resulting in the formation of arginine. In the presence of arginase, this decomposes to urea and ornithine. Thus the ornithine may be used over and over again, producing many times its molecular equivalent of urea. Ornithine may therefore be considered as playing the rôle of a coenzyme. These reactions may be represented as follows:



[4] *Z. physiol. Chem.*, **210**, 33 (1932). [5] *Ann. Rev. Biochem.*, **5**, 247 (1936).

Inasmuch as the fourth step depends on the presence of arginase, this theory explains the non-formation of urea in birds, in the livers of which this enzyme is lacking.

It has not been proved that Krebs' hypothesis applies to the *in vivo* production of urea. London [6] could find no evidence in its support in a series of experiments in which NH_4Cl, with and without ornithine, citrulline, or arginine, were injected into the portal vein of dogs and blood removed from the hepatic vein at intervals for analysis. None of these compounds produced an increase above that obtained with NH_4Cl alone.

That the ammonia liberated in the deamination of amino acids and other substances such as taurine, sacrosine, and asparagine is available for urea production has been shown by Krebs [7] in a series of experiments in which these compounds were added to liver tissue (rat). With a few exceptions (*d*-isoleucine, *l*-aspartic acid, glutamic acid, cystine, phenylalanine), the addition of amino acids resulted in a measurable increase in urea formation.

Function of the Liver in the Production of Urea. The site of the formation of urea has been the subject of numerous investigations. All possible conclusions have been reached, namely (*a*) that urea is formed exclusively in the liver, (*b*) that urea is formed by all the tissues, but chiefly by the liver, (*c*) that all the tissues contribute to the formation of urea and that the liver plays no special rôle in this regard. The experimental evidence upon which these views are based has been reviewed by Bollman, Mann, and Magath.[8]

These investigators studied the effect of complete removal of the liver on urea formation in more than 90 dogs. In every case where urine was secreted after the operation, it was found to contain much less urea than normally. The urea content of the blood and tissues was likewise diminished very markedly. Assuming that no urea was being formed because of the absence of the liver, it was reasonable to argue that, if the excretion of urea were prevented in some way, the urea of the blood and tissues would neither increase nor decrease but remain constant. Bollman and his associates were able to prove the correctness of this assumption in a very satisfactory manner. From certain dogs they removed both kidneys and the liver simultaneously. Because of the removal of the kidneys and the resulting anuria, there would have followed a progressive accumulation of urea in the system if any were being manufactured. Their results showed very definitely, however, that the blood urea remained at a constant level after the operation. In other series of experiments, they removed both kidneys and, after a given interval, during which there occurred a progressive increase of urea in the blood, the liver as well. Immediately after the second opera-

[6] London *et al.*, *Z. physiol. Chem.*, **227**, 233 (1934); **246**, 106 (1937).
[7] *Ibid.*, **217**, 191 (1933).
[8] *Am. J. Physiol.*, **69**, 371 (1924); **92**, 92 (1930).

tion, the increase in urea ceased and its concentration remained at a fairly constant level during the remainder of the experiment. It seemed obvious, therefore, that the production of urea in the body of the dog is entirely dependent on the presence of the liver, since urea formation ceases completely as soon as the liver is removed.[9]

Kisch [10] has reported extra-hepatic urea production in selachians, part of the evidence being based on experiments with dehepatized torpedo rays. Inasmuch as arginase is widely distributed in fishes and especially in selachians, of which the dogfish is the example most completely studied by Hunter and Dauphinee (see p. 385), this result is in harmony with Krebs and Henseleit's theory of urea formation (p. 411).

One may recall in this connection the classical experiment of Minkowski,[11] who, on extirpating the livers of geese, discovered that the uric acid content of the urine was markedly diminished, being replaced by ammonia. These observations indicated that the tissues contributed little if anything to the conversion of ammonia into uric acid. The formation of uric acid in birds, as has already been pointed out, is the analogue of urea formation in mammals.

Ammonia. The normal daily excretion of ammonia in the urine is quite appreciable, being usually in the neighborhood of 0.7 gram. As contrasted with this is the exceedingly low content of ammonia in the blood.[12] It is, of course, conceivable that the kidney may be able to concentrate ammonium salts to a greater extent than other urinary

[9] Rabinowitch (*J. Biol. Chem.*, **83**, 333 [1929]) has reported an unusual case of acute yellow atrophy of unknown origin in which the involvement of the liver was so severe that on *post mortem* examination it looked as though all the glandular epithelium had disappeared, leaving the framework only. Microscopic study of many sections revealed only isolated liver cells, and the staining properties of even these were poor. Under these circumstances the liver must have been practically without function. The kidneys were likewise extensively damaged so that there was almost complete suppression of urine secretion. Before death this patient was in a condition not unlike that of the experimental animals, used by Bollman, Mann, and Magath, in which both the liver and kidneys had been removed. The biochemical findings were likewise similar. The amount of urea found in the urine was practically negligible both on account of the low concentration (0.07 per cent) and the exceedingly small volume (not more than 20 cc. per 24 hours, according to a personal communication). The blood-sugar concentration was 0.046 per cent before and 0.03 per cent after fermentation, which shows that only 16 mg. of glucose was present in 100 cc. of blood. There was no urea in the blood. On the contrary, the amino acid nitrogen concentration was very high, namely 216 mg. per 100 cc., which shows that there was marked retention of amino acids due to impaired renal function and practically no conversion of these into urea. These findings, unique in the literature, confirm the conclusions of Bollman, Mann, and Magath that the liver is the site of urea formation.

[10] *Biochem. Z.*, **225**, 197 (1930).

[11] *Arch. Exptl. Path. Pharm.*, **21**, 41 (1886).

[12] The ammonia concentration of the blood has usually been given as less than 0.05 mg. per 100 cc. According to Folin, this low estimate is due to incomplete recovery and that approximately 0.1 mg. is the more nearly correct normal value (*J. Biol. Chem.*, **97**, 141 [1932]). See also Van Slyke and Hiller, *ibid.*, **102**, 499 (1933).

constituents, but even granting this, there would still be many facts left unexplained. The ammonia elimination may be modified, especially by the intake of acids and fixed bases. The former causes an increase in ammonia elimination, the latter a decrease. Acidosis is characterized by a high ammonia output in the urine. The significance of these changes has been determined through the study of the acid-base equilibrium in conditions of acidosis. The organism can apparently afford to lose considerable quantities of ammonia much better than it can afford to lose fixed bases (K, Na, Ca). Therefore, any ammonia which may be formed for the purpose of neutralizing acid spares an equivalent amount of fixed base. However, inasmuch as there is so little ammonia in the circulation, it can hardly be considered to have a significant rôle in the neutralization of acids transported in the blood. Whatever part it does play in the neutralization of acid may therefore be related to the function of the kidney.

The doctrine that urinary ammonia originates in the kidney has been largely advanced by Nash and Benedict.[13] These investigators have contended that, if the kidneys functioned merely in excreting ammonia formed in other tissues, the effect of total nephrectomy or of tying off the ureters would be an accumulation of ammonia in the blood. Instead, Nash and Benedict found that these surgical manipulations produced no change in the ammonia content of the blood, although there was abundant evidence of retention of other non-protein nitrogenous constituents. They were also able to show that the blood collected from the renal vein in dogs contained on an average twice as much ammonia as was present in blood collected from other sources, such as the vena cava and carotid artery. It would appear, therefore, that the kidney, instead of excreting ammonia from the blood, actually forms the ammonia which it excretes, and in addition contributes a small amount of ammonia to the blood.[14]

Nash and Benedict were led to the conclusion that urea is the most probable precursor of urinary ammonia, although they did not exclude the possibility that the ammonia may be derived from amino acids reaching the kidney. On the other hand, Mann and Bollman considered the source to be practically limited to urea. It will be recalled that in hepatectomized dogs there is a cessation of urea production. Mann and

[13] *J. Biol. Chem.*, **48**, 463 (1921); **69**, 381 (1926); Benedict and Nash, *ibid.*, **82**, 673 (1929); Nash and Williams, **94**, 783 (1932); *J. Pharmacol. Exptl. Therap.*, **45**, 487 (1932).

[14] These observations have been confirmed by Loeb, Atchley, and E. M. Benedict (*J. Biol. Chem.*, **60**, 491 [1924]), who likewise observed that blood from the renal vein of the dog contained more ammonia than blood from the vena cava or femoral artery. In rabbits the relation is somewhat different, as might be expected from the fact that these animals excrete an alkaline urine which contains only traces of ammonia. Accordingly, little ammonia is formed in the kidney and hence its content in the blood of the renal vein does not differ appreciably from the concentration in the general circulation.

Bollman [15] observed that, when the urine of dehepatized dogs became extremely low in urea, the ammonia content was likewise diminished. If at this time urea was injected intravenously, there resulted a definite increase in ammonia excretion, an effect which was not produced by the injection of amino acids. It was therefore concluded that urea is the precursor of urinary ammonia.

This view has been challenged, however. It has been shown, for example, in perfusion experiments with dog's kidney, that there is an increase in the ammonia content of the perfusate after the addition of glycine to the perfusion fluid.[16] According to Krebs,[17] amino acids are deaminized more rapidly by kidney than by liver. No evidence was found for the conversion of urea into ammonia by slices of kidney, which accords with the fact that the kidney contains no urease. Support has therefore shifted to the view that the main precursors of urinary ammonia are the amino acids.

It is interesting to note that, in uranium nephritis [18] (and this has also been observed in clinical nephritis), there is a marked reduction in the excretion of ammonia.

Ammonia from Adenylic Acid. The occurrence of adenylic acid in muscle has been referred to in other connections. Since its discovery by Embden,[19] it has been found in other tissues. Its presence in the kidney necessitates its consideration as precursor of urinary ammonia, but as it occurs in relatively small amount, it is obviously of much less importance than the amino acids.

In muscle, however, it plays a much more significant rôle. As it exists in resting muscle, most if not all of the adenylic acid is combined with two additional labile molecules of phosphoric acid. The combination is described by certain authors as adenylpyrophosphoric acid (adenosinepyrophosphate, etc.); by others it is designated adenyltriphosphoric acid (adenosinetriphosphate, etc.). Adenyldiphosphoric acid has also been isolated from muscle. Deamination of adenylic acid and of the triphosphate proceeds simultaneously and at about the same rate, the products formed being inosinephosphate (inosinic acid) and inosinetriphosphate, respectively. The former compound (p. 431) has been known for a long time, having been discovered by Liebig in 1847, but its significance was unappreciated until the important researches of Embden [20] and of Parnas [21] and their associates made it clear that the deamination of adenylic acid is related to muscular activity.

[15] *Am. J. Physiol.*, **85**, 390 (1928); **92**, 92 (1930).

[16] A. Bornstein and G. Budelmann, *Biochem. Z.*, **218**, 64 (1930); see also A. Patey and E. B. Holmes, *Biochem. J.*, **24**, 1564 (1930).

[17] *Z. physiol. Chem.*, **217**, 191 (1933); **218**, 157 (1933).

[18] B. M. Hendrix and M. Bodansky, *J. Biol. Chem.* **60**, 657 (1924).

[19] *Z. physiol. Chem.*, **167**, 137 (1927).

[20] *Ibid.*, **179**, 149 (1928).

[21] J. K. Parnas and W. Mozolowski, *Biochem. Z.*, **184**, 399 (1927); Parnas, *ibid.*, **206**, 16 (1929).

Under anaerobic conditions adenylic acid (using this term to include the triphosphate) is converted into inosinic acid, the latter accumulating in the muscle. However, when oxygen is available and muscle is working without fatigue, large amounts of ammonia may accumulate, but there is no decrease in adenylic acid. The restoration of the adenylic acid is apparently due to the re-amination of the inosinic acid. According to Parnas, the reaction adenylic acid → inosinic acid is irreversible, the ammonia being derived from amino acids. In accordance with this view, the changes during contraction and recovery may be represented as follows:

1. Contraction.
   Adenylic acid → inosinic acid + NH_3

2. Recovery.
   Inosinic acid + O_2 + amino-acid *X* → adenylic acid + deaminized *X*

According to Parnas and Lutwak-Mann,[22] adenosinetriphosphoric acid is the principal, but not the only, source of ammonia. They have submitted data showing that the loss of ammonia from adenylic acid precedes the deamination of some unknown ammonia-yielding compound. The nature and physiological significance of this source of ammonia remain to be determined.[23]

CREATINE AND CREATININE

Creatine is methylguanidineacetic acid. It is widely distributed in animal tissues and is especially abundant in skeletal muscle. Calculations based on chemical analyses of the tissues indicate that the adult human body contains on an average about 100 grams of creatine, most of which is present in the muscles, where its concentration normally averages about 450 mg. per 100 grams. Heart muscle contains approximately 200 mg. per cent of creatine, more being present in the left than in the right ventricle.[24]

[22] *Biochem. Z.*, **278**, 11 (1935).

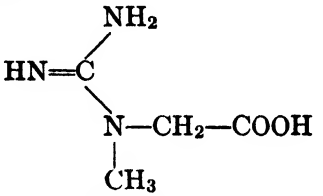
[23] There is probably no relation between the ammonia formed in muscle and that which is excreted in the urine. In attempting to associate the two, however, Bliss (*J. Biol. Chem.*, **81**, 137 [1929]; *J. Pharmacol.*, **40**, 171 [1930]; **44**, 397 [1932]), has postulated that the ammonia is transported as an amide in combination with the blood proteins and that this complex is enzymically deaminized in the kidney, resulting in the liberation of ammonia which in turn combines with an acid radical (such as lactate) and is excreted in the urine. A criticism of this view is to be found in the papers by Benedict and Nash, and Nash and Williams.[13]

[24] For data concerning the creatine content of the human heart, see D. W. Cowan, *Am. Heart J.*, **9**, 378 (1934); D. P. Seecof, C. R. Linegar and V. C. Myers, *Arch. Internal Med.*, **53**, 574 (1934); M. Bodansky, J. F. Pilcher and V. B. Duff, *ibid.*, **59**, 232 (1937). Creatine occurs in the testes, liver, kidney, pancreas, spleen, thyroid, thymus, and brain. It is of particular interest to note that in mammals the testes

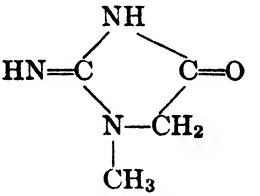
Creatinine is the anhydride of creatine and is a normal constituent of urine. From 1 to 2 grams of this substance are excreted daily by an adult man or woman, this amount being constant from day to day, especially in males. Normally, creatine is not found in the urine of male adults. It is occasionally found in the urine of females, and in that of children it is a normally occurring constituent. As first shown by Folin,[25] the elimination of creatinine is not influenced by the amount of protein in the diet, but is apparently a measure of endogenous protein metabolism.

When heated in acid solution, creatine is converted into creatinine; in an alkaline solution the reverse change takes place. The close chemical relationship between creatine and creatinine and the ease with which one is changed into the other in the laboratory would suggest that they are similarly affiliated physiologically. The subject of creatine and creatinine metabolism has engaged the efforts of numerous biochemists, and yet our knowledge of it is still incomplete.

The relation of creatine to creatinine is indicated by the following formulas:

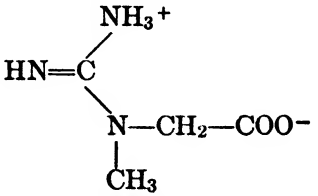


Creatine



Creatinine

It is believed, however, that free creatine doubtless exists in the form of an internal salt, represented as follows:



Creatine

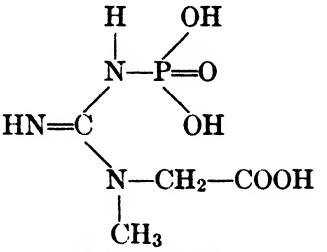
Creatinephosphate (Phosphocreatine). Eggleton and Eggleton,[26] on the basis of certain analyses of phosphate in muscle, reached the im-

stand next to skeletal and cardiac muscle in creatine content. According to Greenwald (Proc. XIV International Physiological Congress, Rome, 1932), the gonads of certain invertebrates, the tunicate *Boltinia*, *Echinurus*, the squid, and the sea-urchin *Arbacia pustulosa*, contain considerable amounts of creatine, much more being present in the male gonads than in those of the female. Greenwald has suggested that creatine may have functions other than those related to muscular contraction.

[25] *Am. J. Physiol.*, **13**, 66 (1905).

[26] *Biochem. J.*, **21**, 190 (1927); *J. Physiol.*, **63**, 155 (1927); *Physiol. Rev.*, **9**, 432 (1929).

portant conclusion that muscle contains a labile (easily hydrolyzable) organic phosphate, which they called *phosphagen*. At about the same time, Fiske and Subbarow,[27] in this country, made a similar observation and showed that the substance in question was composed of creatine and phosphoric acid. Later studies confirmed these observations and indicated that creatinephosphate has the following constitution:



Creatinephosphate

Stimulation of muscle is associated with the decomposition of creatinephosphate. This compound is so unstable that, in experiments involving its analysis, special precautions must be taken to prevent or minimize excitation of the muscle during removal. To avoid creatinephosphate hydrolysis after removal, the tissue is promptly frozen in liquid air. Freezing of tissue *in situ* before removal for analysis has also been employed.

Traumatic damage of muscles causes unusually rapid cleavage of the creatinephosphate which is present. Rapid hydrolysis may be produced also by acid and alkali.

In resting muscle the greater part of the creatine occurs as phosphocreatine; indeed, it has even been questioned whether free creatine is present in significant amounts. The molar concentration of the total free and labile phosphate in muscle (0.029–0.036) is greater than that of the creatine (0.025–0.03), so that there is the possibility of all the creatine being combined as phosphate. However, even in resting muscle a certain amount of the creatine is not combined with orthophosphoric acid (H_3PO_4) and is diffusible. Eggleton [28] has estimated that in resting muscle (frog sartorius) about 80 per cent of the creatine is present as phosphocreatine.

Our views concerning the rôle of creatinephosphate in muscle metabolism have changed significantly in the last few years.[29] On the

[27] *Science*, **65**, 401 (1927); *J. Biol. Chem.*, **81**, 629 (1929).

[28] *J. Physiol.*, **70**, 294 (1930).

[29] Soon after the discovery of phosphocreatine, it was shown by Meyerhof (*Biochem. Z.*, **191**, 106 [1927]), that its hydrolysis either by acid or enzyme is accompanied by the liberation of considerable heat (11,000 to 12,500 calories per mole). Then followed the observation of Nachmansohn (*Ibid.*, **196**, 73 [1928]), that in muscular activity the phosphocreatine first broken down is rapidly restored even in the

basis of certain observations it was supposed that creatinephosphate hydrolysis provided the energy for muscular contraction and that its resynthesis occurred at the expense of energy derived from the breakdown of adenyltriphosphate. It is now believed that phosphocreatine probably serves as a source of phosphate which it passes on to adenylic acid. The latter also functions as a coenzyme in dephosphorylation of phosphopyruvic acid (p. 329). The phosphate from both sources goes into the formation of adenyltriphosphate, which in turn gives up its labile phosphoric acid to glycogen for the esterification of hexose and to creatine for the restitution of the creatinephosphate. The reaction between adenyltriphosphate and creatine is therefore a reversible one. According to Lehmann,[30] this bimolecular reaction may be represented as follows:[31]

Adenylpyrophosphate + creatine $\rightleftarrows$ adenosinediphosphate + creatinephosphate

Adenosinediphosphate + creatine $\rightleftarrows$ adenylic acid + creatinephosphate

The energy change involved is comparatively small. During anaerobic contraction the reactions:

absence of oxygen. The source of the energy required for the resynthesis was at first obscure.

Two years later Lundsgaard (*Ibid.*, **217**, 162; **220**, 1, 8; **227**, 51 [1930]; **230**, 10; **233**, 322 [1931]), described an experiment in which it was shown that frog's muscle poisoned with iodoacetic acid may contract, but that no lactic acid is formed in the process. This was an astounding discovery, as it was contrary to the prevailing conception that the energy for contraction is obtained from the formation of lactic acid. Instead of the glycogen → lactic acid transformation, the contraction of the poisoned muscle was evidently associated with the breakdown of creatine phosphate. The theory was therefore advanced that the cleavage of phosphocreatine is the primary change and supplies the energy in the normal contraction of muscle.

[30] *Biochem. Z.*, **286**, 336 (1936).

[31] The synthesis of creatinephosphate in muscle is enzymatic and is coupled with the so-called Parnas reaction, which refers to the transfer of phosphate from phosphopyruvic acid (p. 329). This may be represented in two stages as follows:

(1) 2 Phosphopyruvic acid + adenylic acid = 2 pyruvic acid + 1 adenosinetriphosphate
(2) 1 Adenosinetriphosphate + 2 creatine = 1 adenylic acid + 2 creatinephosphate

O. Meyerhof, W. Schulz, and Ph. Schuster have recently submitted evidence (*Biochem. Z.*, **293**, 309 [1937]) that, in addition to the above reaction, creatinephosphate is synthesized enzymatically in the oxidation-reduction phase of lactic acid production:

(1) 1 H_3PO_4 + 1 creatine + 1 triosephosphate + 1 pyruvic acid = 1 creatinephosphate + 1 phosphoglyceric acid + 1 lactic acid
(2) 1 creatine + 1 phosphoglyceric acid = 1 creatine phosphate + pyruvic acid

These two reactions may be combined

1 H_3PO_4 + 2 creatine + 1 triosephosphate = 2 creatinephosphate + 1 lactic acid

(1) Adenylpyrophosphate → adenylic acid + $2H_3PO_4$,

and

(2) Adenylpyrophosphate + glycogen → 2 adenylic acid + 2 hexosediphosphate, are each accompanied by the liberation of 24,000 calories per mole. On the other hand, the reaction:

2 Creatinephosphate + 2 adenylic acid → adenylpyrophosphate + 2 creatine, being endothermic, absorbs about 1000 calories per mole. In the restitution of the phosphocreatine during anaerobic recovery the same amount of energy is liberated.[32]

It has been reported that white muscle is richer in creatinephosphate than red muscle.[33] The former is rapidly contractile; the latter contracts much more slowly. It has been inferred that in the slowly contracting red muscle there is more time for the restitution of the phosphagen, so that the amount of it can be less.

Phosphocreatine occurs in nerve tissue, where its properties are very similar to those in muscle.[34] It has been isolated from brain by Kerr.[35] When nerve is deprived of oxygen the phosphocreatine decomposes very rapidly. The rate of decomposition is further accelerated if the nerve is treated with a monohalogen derivative of acetic acid. The electric organ of the torpedo ray contains phosphocreatine.[36]

In the muscle of crustaceans, Meyerhof and Lohmann [37] found phosphoarginine (arginine phosphate), the properties and functions of which appear to be analogous to those of phosphocreatine. The distribution of this phosphagen is not restricted to the Crustacea, however, for it has been determined in coelenterates, platyhelminths, annelids, cephalopods, echinoderms, and urochords (*Ascidia*). In certain echinoderms and in the hemichords, both argininephosphate and creatinephosphate are present, whereas in the cephalochords (*Amphioxus*) and Craniata (vertebrates) creatinephosphate alone has been found so far. In his monograph, Meyerhof refers to creatinephosphoric acid as the vertebrate phosphagen (*Wirbeltierphosphagen*), whereas argininephosphoric acid is described as the invertebrate phosphagen (*Wirbellosenphosphagen*).[38]

[32] For more detailed outline of the chemical reactions and energy exchanges during anaerobic contraction and recovery, the reader is referred to D. M. Needham, *Ann. Rev. Biochem.*, **6**, 395 (1937).

[33] D. Ferdmann and O. Feinschmidt, *Z. physiol. Chem.*, **178**, 173 (1928); A. Palladin, and S. Epplebaum, *ibid.*, **178**, 179 (1928).

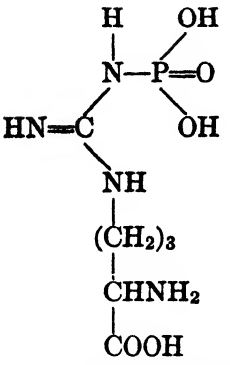
[34] R. W. Gerard, "Nerve Metabolism," *Physiol. Rev.*, **12**, 469–592, especially 497 (1932).

[35] *J. Biol. Chem.*, **110**, 625 (1935).

[36] B. Kisch, *Biochem. Z.*, **225**, 183 (1930).

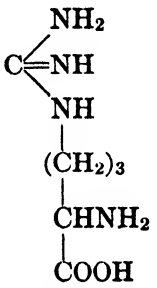
[37] *Naturwissenschaften*, **16**, 726 (1928).

[38] F. Kutscher and D. Ackermann, *Ann. Rev. Biochem.*, **2**, 355 (1933); A. Arnold and J. M. Luck, *J. Biol. Chem.*, **99**, 677 (1933). O. Meyerhof, "Die chemischen Vorgänge im Muskel," Berlin, 1930, p. 93. See also E. Baldwin, "An Introduction to Comparative Biochemistry," Chapter V.

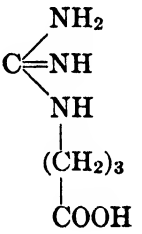


Phosphoarginine
(Argininephosphoric acid)

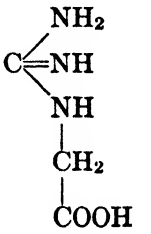
Origin of Creatine. It is reasonable to look to arginine as a possible precursor of creatine, for it is the only one of the constituents of protein which possesses a guanidine group. As early as 1905 Czernecki [39] proposed the theory, later adopted by Knoop[40] and Neubauer,[41] that arginine gives rise to guanidinobutyric acid and in turn to guanidinoacetic acid, from which by methylation creatine is formed. The following formulas indicate the relation of these compounds to one another:



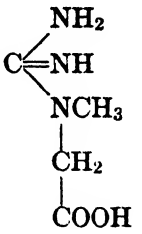
Arginine



Guanidino-butyric acid



Guanidinoacetic acid (guanidoacetic acid, glycocyamine)



Methylguanidino-acetic acid (creatine)

However, proof of such a metabolic relationship between arginine and creatine is lacking, although the conversion of guanidinoacetic acid into creatine is fairly generally accepted.

The inconsistency of the evidence for arginine as a creatine precursor may be illustrated by a few examples. In the pig, Gross and Steenbock [42] observed that arginine administered orally augments creatine excretion. Hyde and Rose,[43] on the contrary, after feeding arginine for

[39] *Z. physiol. Chem.*, **44**, 294 (1905).
[40] *Ibid.*, **67**, 489 (1910).
[41] *Handlexikon d. Biochem.*, **4**, 386 (1911).
[42] *J. Biol. Chem.*, **47**, 33 (1921).
[43] *Ibid.*, **84**, 535 (1929).

as long as 8 weeks found no evidence of its conversion into creatine or creatinine in man. A similar result was obtained by Grant, Christman, and Lewis,[44] who fed arginine to a dog for 35 days and failed to influence the urinary excretion of either creatine or creatinine. That creatine formation is independent of the amount of protein fed,[45] or of arginine contained in the diet,[46] has also been demonstrated in rats. Nor is there any evidence for the transformation of phosphoarginine into phosphocreatine, creatine, or creatinine.[47] In reviewing the evidence for the origin of creatine from arginine, Hunter [48] states, "So large a body of almost purely negative evidence leads one rather forcibly to suspect that, if creatine is related to arginine at all, its mother substance must be not the free amino-acid, but the still combined arginine of the muscle or other tissue protein."

In view of the foregoing it is of interest that Fisher and Wilhelmi [49] recently reported perfusion experiments on the isolated rabbit heart, in which an increase in total creatinine (creatine plus creatinine) was obtained on adding arginine to the perfusate. The increase in creatinine corresponded almost precisely to the amount of arginine which disappeared. This applied only to the heart of post-pubertal animals. No increase was observed in the hearts of prepubertal rabbits.

Glycine. The idea that glycine may be a precursor of creatine grew out of the observation that in persons with progressive muscle dystrophy feeding glycine induced a marked increase in the output of creatine. In normal persons this response is either absent or very slight.[50]

In order that the extra creatine excreted may be more definitely related to synthesis, it would be necessary to rule out conclusively other effects that glycine may have either in the human subject or in the experimental animal. Other factors, such as stimulation of cellular metabolism, increased renal function, or a "washing out" of free creatine from the tissues, may conceivably lead to an increased output of creatine. It may be mentioned incidentally that in progressive muscle dystrophy the ability to synthesize glycine is normal.[51]

Although the evidence for the formation of creatine from glycine is at most inconclusive, certain comments by Kutscher and Ackermann [52]

[44] *Proc. Soc. Exptl. Biol. Med.*, **27**, 231 (1929).

[45] A. Chanutin, *J. Biol. Chem.*, **89**, 765 (1930).

[46] C. E. Meyer and W. C. Rose, *ibid.*, **102**, 461 (1933).

[47] D. M. Brown and J. M. Luck, *Proc. Soc. Exptl. Biol. Med.*, **29**, 723 (1932).

[48] A. Hunter, "Creatine and Creatinine," Longmans, Green & Co., London and New York, 1928, p. 227.

[49] *Biochem. J.*, **31**, 1131 (1937).

[50] E. Brand, M. M. Harris, M. Sandberg, and A. I. Ringer, *Am. J. Physiol.*, **90**, 296 (1929); Brand, Harris, Sandberg, and M. M. Lasker, *J. Biol. Chem.*, **87**, ix (1930); Brand and Harris, *ibid.*, **92**, lix (1932); *J. Am. Med. Assoc.*, **101**, 1047 (1933); A. T. Milhorat, *Deut. Arch. klin. Med.*, **174**, 487 (1933); see also M. Bodansky, *J. Biol. Chem.*, **115**, 641 (1936).

[51] I. K. Freiberg and E. S. West, *J. Biol. Chem.*, **101**, 449 (1933).

[52] *Ann. Rev. Biochem.*, **5**, 453 (1936).

are of significance in this connection. They point out that the guanidine derivative *asterubin* (discovered by Ackermann in certain species of starfish) is obviously a derivative of taurine. What is more interesting is that asterubin often occurs in the presence of free taurine. From this it seems likely that asterubin is formed by the introduction of a guanidine residue ("guanylation") into taurine, and methylation. Kutscher and Ackermann infer that guanylation and methylation of glycine would yield creatine. The assumption is that the organism synthesizes the guanidine nucleus *de novo*.

Choline and betaine have been described as precursors of creatine (Riesser).[53] Abderhalden and Buadze[54] believe histidine to be a precursor. Beard and Barnes[55] have described experiments in which very striking increases in muscle creatine occurred following the administration of a variety of compounds (amino acids, protein, etc.).

Fate of Creatine and Its Conversion into Creatinine. At the time when Folin[56] published his classical study on protein metabolism, the conversion of creatine into creatinine by the body was not questioned. Folin observed, however, that feeding creatine had no effect on the excretion of creatinine. Taken in small amounts, creatine was retained entirely; when larger quantities were ingested, only a part was retained, the remainder being excreted unchanged. As a result of these observations, Folin came to the conclusion that the organism did not possess the power of converting creatine into creatinine and that these substances were independent of each other in metabolism. Folin regarded creatine as a food and creatinine as a waste product.

Since their publication, Folin's results have been confirmed and denied by numerous workers. In 1916, Rose and Dimmitt[57] furnished evidence in support of the view that creatine is convertible into creatinine. These workers found that the ingestion of large doses of creatine in man caused an appreciable increase in the output of creatinine. Thus, in certain of their experiments, the ingestion of 10 grams of creatine caused an increase of 0.26–0.34 gram of creatinine, whereas the increases observed after taking 20 grams of creatine were between 0.30 and 0.49 gram. The greater part of the creatine, however, was excreted unchanged. The ingestion of creatinine, on the contrary, was not followed by the appearance of creatine in the urine, from which it may be inferred that in the body the reaction creatine → creatinine is an irreversible one.

Benedict and Osterberg[58] studied the effects of prolonged feeding of creatine. In one experiment they fed a dog a small quantity of creatine daily for a period of 70 days. Thus a total amount of 32.9

[53] *Z. physiol. Chem.*, **86**, 415 (1913); **90**, 221 (1914).
[54] *Ibid.*, **200**, 87 (1931).
[55] *J. Biol. Chem.*, **94**, 49 (1931–32).
[56] "Hammarsten's Festschrift," Upsala, part 3 (1906).
[57] *J. Biol. Chem.*, **26**, 345 (1916).
[58] *Ibid.*, **56**, 229 (1923).

grams of creatine (expressed as creatinine) was given. The urine was analyzed daily for a long period before creatine administration was begun to establish the dog's normal output of creatinine and creatine (in this case creatine was absent from the urine normally). The urine was analyzed daily during the experimental period and the analyses were continued for 7 weeks after the creatine feedings had been discontinued. Creatinuria was not observed until the tenth day after the administration of creatine was begun. From this time on, increasing amounts of creatine were present in the urine, the creatinuria continuing until the day after creatine feeding was stopped. Of the 32.9 grams of creatine fed during the 70 days, a total of 13 grams of creatine was recovered. Accordingly, 19.9 grams of creatine had been retained by the tissues. An increased daily excretion of creatinine became manifest about one week after the creatine administrations were instituted and this continued for the duration of the experiment, including the after period of 7 weeks. The extra creatinine eliminated was 5.8 grams, or 29.1 per cent of the creatine retained. The difference of 14.1 grams could not be accounted for either as extra urinary creatine or creatinine.

These results are of importance because they establish (1) that creatine is converted into creatinine, (2) that this conversion is not a direct process, but apparently involves a preliminary storage of the creatine in the tissues, (3) that creatinine is probably only one of the end-products of creatine metabolism, and that a proportion of the creatine may follow a different metabolic path.

Results similar to those of Benedict and Osterberg have been obtained in rats by Chanutin [59] and in man by Rose, Ellis, and Helming.[60] More recently, Chanutin and Silvette [61] have approached more directly the question of why administered creatine is not fully recovered in the urine either as extra creatine or creatinine. They injected creatine into completely nephrectomized rats and analyzed the various tissues for creatine. They showed that larger amounts were stored in the liver and muscles than elsewhere and moreover that not all of the creatine administered could be recovered in the tissues. In this way Chanutin and Silvette have demonstrated that creatine may be destroyed by the organism.

With the advance of knowledge it has become increasingly clear that creatine is probably the immediate precursor of creatinine, but that its metabolism is not restricted to this reaction. Creatinine is to be regarded as an end-product, whereas creatine is doubtless an essential tissue constituent with a special function.[62]

[59] *Ibid.*, **67**, 29 (1926).

[60] *Ibid.*, **77**, 171 (1928).

[61] *Ibid.*, **85**, 179 (1929); **89**, 765 (1930); see also **75**, 549 (1927); **80**, 589 (1928); Chanutin and H. H. Beard, *ibid.*, **78**, 167 (1928).

[62] It should not be assumed that this view is universally shared. See for example, Thomas, Milhorat, and Techner, *Z. physiol. Chem.*, **214**, 121 (1933). Catherwood and Stearns, *J. Biol. Chem.*, **119**, 201 (1937), believe that creatine and creatinine represent different phases of muscle metabolism not closely interrelated.

Creatinuria. Creatine is not present normally in the urine of the male adult but does occur as a normal constituent in the urine of children of both sexes before the age of puberty. Creatinuria is likewise manifested in starvation, muscular diseases, exophthalmic goiter, eclampsia, and diabetes. As we shall see, not all forms of creatinuria can be referred to the same fundamental cause, although it is evident that all are due either to incomplete storage of creatine or to incomplete conversion of creatine into creatinine.

Exogenous and Endogenous Creatinuria. In the first place, it is important to consider whether all forms of creatinuria are endogenous in origin. On this point there is much difference of opinion. According to one group of workers, notably Denis,[63] the ingestion of large quantities of protein may cause creatinuria in cases where it is absent; or increase it where it already exists, whereas the reverse effect is obtained upon a minimum protein intake.

Rose,[64] on the other hand, was unable to induce creatinuria by feeding large quantities of protein to men and women. In some of his experiments, the protein intake was so high as to result in a nitrogen excretion in the urine of as much as 35 grams. Nevertheless, there was no evidence of creatinuria in these individuals.

Creatine disappears from the urine of pigs kept on a diet practically devoid of protein; but containing a sufficient quantity of carbohydrate to meet the calorific requirements (Terroine [65]). Under these conditions the creatinine output is remarkably constant.

Hunter [48] has pointed out that the excretion of creatine on a high protein intake need not necessarily be interpreted as proof of the exogenous origin of creatine. The well-known effect of proteins in stimulating cellular metabolism (specific dynamic action) has been held responsible (Lewis, Dunn, and Doisy) [66] for the increased excretion of endogenous uric acid in experiments in which excessive amounts of proteins and amino acids were fed. It may likewise have been the cause of the increased creatine formation in those cases where this has been observed. Accordingly, creatine and creatinine metabolism is to be regarded as a phase of endogenous metabolism. The only obvious exception to this occurs when creatine as such is ingested.

Creatinuria in Children, Women, etc. Infants and children normally excrete creatine in addition to creatinine, as was first shown by Rose.[67] Several explanations have been offered for this phenomenon. It has been suggested that creatinuria in children may be due to the fact that they have less ability to retain creatine because their musculature is

[63] *J. Biol. Chem.*, **29**, 447 (1917); **30**, 47, 189 (1917); **31**, 561 (1917); **37**, 245 (1919).
[64] *Ibid.*, **34**, 601 (1918).
[65] *Bull. soc. chim. biol.*, **15**, 23, 42 (1933).
[66] *J. Biol. Chem.*, **36**, 9 (1918).
[67] *Ibid.*, **10**, 265 (1911).

less developed and proportionately less abundant than in adults. However, Catherwood and Stearns [62] are of the opinion that immaturity of muscle is apparently not a factor in determining the amount of creatine excretion in infancy (birth to 1 year of age). They were unable to recognize any relation of creatinuria either to the amount of creatine in the diet or to any phase of nitrogen metabolism. It is concluded that thyroid function is of importance in influencing either directly or indirectly the level of creatine excretion in infants.

In women, after puberty, creatinuria is intermittent, except during pregnancy, when it is continuous. Whether creatinuria in women is to be related, as in children, to deficient muscular development or sex-glandular control, or to some other cause, is a matter that we are not able to decide at present, although the indications are that even muscularly well-developed women exhibit an occasional creatinuria. This indicates that sex is a probable factor, a view strengthened by the observations that creatinuria occurs in eunuchs [68] and, as in prepubescent boys, may be readily induced by the oral administration of small amounts of creatine in aged men and others with extinguished testicular function.[69]

Creatinuria in Starvation and Carbohydrate Deprivation. From the work of Brentano [70] and others it would seem that the ability of muscle and perhaps of other tissues to retain creatine depends on the glycogen reserve. If for any cause this is lowered there follows an increase in creatine excretion. Starvation is one of the conditions in which creatinuria is a prominent feature, occurring in every species of mammal that has been observed. Starvation creatinuria may be abolished by the administration of carbohydrate. Protein is nearly as effective in this respect, either because of its property as a tissue sparer, or by providing glucose and glycogen precursors.[71]

That a relationship exists between creatine-creatinine metabolism and carbohydrate metabolism is also indicated by the fact that creatinuria is characteristic of many conditions of deficient carbohydrate utilization (diabetes, phlorhizin glycosuria) as well as of conditions in which there is apparently a deficiency in glycogenic function. In the latter group are included various forms of hepatic insufficiency, such as occur in eclampsia, phosphorus and chloroform poisoning, and carcinoma of the liver. It is to be noted, however, that in none of these can we exclude entirely the factor of exaggerated endogenous or tissue metabolism.

[68] B. Read, *ibid.*, **46**, 281 (1921).

[69] L. Remen, *Z. exptl. Med.*, **80**, 238 (1931); F. Lasch, *ibid.*, **81**, 681 (1932); compare with I. Schrire and H. Zwarenstein, *Biochem. J.*, **26**, 118, 1886 (1932); **27**, 1337 (1923); **28**, 356 (1934).

[70] *Arch. exptl. Pathol. Pharmakol.*, **155**, 21 (1930); **163**, 156 (1931–2); *Z. klin. Med.*, **120**, 249 (1932).

[71] W. C. Rose, F. W. Dimmitt, and P. N. Cheatham, *J. Biol. Chem.*, **26**, 339 (1916).

It has been found that rats fed thyroid substance, or injected with thyroxine over long periods, excrete large amounts of creatine. Although the increased creatinuria is partly associated with an augmented nitrogen metabolism, it is also accompanied by a marked loss of creatine from the tissues, especially heart and skeletal muscle. The creatine reserve is gradually restored upon discontinuing the administration of thyroid or thyroxine.

These observations are perhaps not unrelated to clinical hyperthyroidism (Graves' disease) in which creatinuria occurs not only after the administration of creatine, but also on a creatine-free diet.[72] This may be due to increased tissue protein destruction, but is more likely related to a reduced ability of the muscle to retain the creatine formed in metabolism. Glycogen depletion, as a factor favoring creatinuria, is probably as important in hyperthyroidism as in starvation. Whatever the explanation may be, the creatinuria is related to the heightened basal metabolism characteristic of the disease, for when this is reduced, as through the administration of iodine, the creatinuria is likewise diminished. That the changes in the muscle, both anatomical and metabolic, occurring in hyperthyroidism resemble those in progressive muscular dystrophy has been emphasized by Shorr and associates.[73] It is of particular interest to note that these investigators regard the muscular weakness in Graves' disease to be the result of a reparable impairment of the phosphocreatine mechanism.

It has been reported that whereas normal children excrete creatine, in those with thyroid deficiency creatinuria is diminished and may even be absent.[74]

Creatinuria in Diseases Affecting the Muscles. In his review of the subject, Hunter[75] refers to the following primary and secondary diseases of the muscular system in which creatinuria is known to occur: progressive muscular dystrophy, amyotonia congenita, progressive muscular atrophy, amyotrophic lateral sclerosis, myasthenia gravis, atrophies resulting from disuse, acute anterior poliomyelitis and other lesions of the spinal cord or motor nerves. In trichinosis, a condition associated with an inflammatory process in the muscles, there is marked creatinuria, as there is in generalized myositis fibrosa. In myotonia congenita there appears to be no abnormality of the creatine-creatinine metabolism. Although the literature on the subject of muscle disease is very extensive, fundamental knowledge is still lacking.

Goettsch and Brown[76] have described a nutritional form of muscular

[72] W. W. Palmer, D. A. Carson, and L. W. Sloan, *J. Clin. Investigation*, **6**, 597 (1928–29); E. J. Kepler and W. M. Boothby, *Am. J. Med. Sci.*, **182**, 476 (1931).

[73] E. Shorr, H. B. Richardson, and H. G. Wolff, *J. Clin. Investigation*, **12**, 966 (1933).

[74] H. G. Poncher, M. B. Visscher, and H. Woodward, *J. Am. Med. Assoc.*, **102**, 1132 (1932).

[75] *Loc. cit.*,[48] p. 218.

[76] *J. Biol. Chem.*, **97**, 549 (1932).

dystrophy in rabbits, characterized by a marked reduction of the creatine content of the muscles. It is of interest to note that low muscle creatine values have been reported in myasthenia gravis [77] and particularly in generalized myositis fibrosa.[78]

Significance of Creatinine. Although there is much indirect evidence that at least the greater part of the substance in the blood giving the Jaffé reaction is identical with creatinine, this has been proved only recently. Experiments designed to ascertain the relation of the chromogenic constituent of the blood to urinary creatinine (comparison of the concentrations of the renal venous and arterial blood) have disclosed that the kidney removes enough of the apparent creatinine of the blood to account for the urinary creatinine. This has led Zacherl [79] to the conclusion that the blood contains preformed creatinine.

Miller and Dubos [80] have shown that the chromogenic substance in human blood is decomposed by two bacterial enzymes, each possessing a high degree of specificity for creatinine, as compared to its action on other Jaffé-reactive compounds. They conclude that creatinine constitutes almost all of the chromogenic material in normal plasma.

Creatinine (or the chromogenic substance reacting like creatinine) is present in very small amounts in the tissues. It is a true waste product and as such is normally removed. In a given individual the elimination of creatinine is constant from day to day, provided the diet is free from creatine and creatinine, present as such. The amount of creatinine excreted daily is independent of the volume of urine, the amount of protein in the diet, and therefore of the total nitrogen metabolism. It is not influenced by the amount of ordinary muscular work (Shaffer [81]). When creatine appears, as in fasting, it is at the expense of creatinine, suggesting incomplete conversion of creatine into creatinine. Under these conditions the output of creatine plus creatinine is equivalent to the amount of creatinine which would have been excreted if there were no creatine. In view of this remarkable constancy in creatine-creatinine metabolism in a given individual it is of interest to inquire concerning the cause of variation in creatinine excretion in different individuals.

The Creatinine Coefficient. The number of milligrams of creatinine excreted in the urine in twenty-four hours per kilogram of body weight

[77] B. W. Williams and C. S. Dyke, *Quart. J. Med.*, **15**, 269 (1922).

[78] M. Bodansky, E. H. Schwab, and P. Brindley, *J. Biol. Chem.*, **85**, 307 (1929–30).

[79] *Z. physiol. Chem.*, **248**, 80 (1937); compare with A. Goudsmit, *J. Biol. Chem.*, **115**, 80 (1936).

[80] *J. Biol. Chem.*, **121**, 447 (1937).

[81] *Am. J. Physiol.*, **22**, 445 (1908); compare K. Kácl, *Biochem. Z.*, **245**, 453 (1932). Shaffer's statement does not agree with the observations of A. B. Light and C. R. Warren, *J. Biol. Chem.*, **104**, 121 (1934). They found that subjects (adolescent males at school) resting in bed excrete far less creatine than while attending classes and engaging in exercise. This was true even when a lower protein diet was given on the day of activity than during the day at rest.

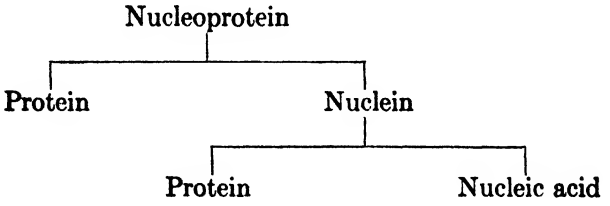
is called the creatinine coefficient. In men, the creatinine coefficient may vary between 18 and 32, with an average of about 24 or 25. In women, lower values are the rule, the average being about 18, with a normal range between 9 and 26. Children have even lower values, ranging between 9 and 17 at 5–13 years (Krause [82]). The more muscular an individual, the higher is his or her creatinine coefficient. Thus, obese or muscularly under-developed men may have a very low creatinine coefficient (as low as 15-18), whereas well-developed muscular women may have as high creatinine coefficients as normal men.[83]

In different animal species, according to Myers and Fine,[84] the creatinine coefficient is related not only to the amount of muscle, but to their creatine content as well. On the contrary, Chanutin and Kinard [85] could establish no such correlation from their experiments on dogs, rabbits, rats, and guinea pigs.

PURINE AND PYRIMIDINE METABOLISM

Under purine and nucleic acid metabolism, we have to consider (1) the fate of ingested nucleoproteins and nucleic acids and (2) the anabolism and catabolism of purines and pyrimidines in the body, whether derived from exogenous or endogenous sources.

Nucleic acids are present in the nuclei of cells. It is not known with certainty that they ever occur in the cytoplasm. According to the prevailing view, the nucleic acids are combined with protein, thus constituting the so-called nucleoproteins. On partial hydrolysis, nucleoproteins yield a protein residue and a protein-nucleic acid complex which has been called nuclein. If the cleavage is carried somewhat further, the nuclein yields protein and nucleic acid as cleavage products. These changes may be represented as follows:



There are two principal nucleic acids. One may be prepared from yeast, but is also present in abundance in wheat embryo. The other is

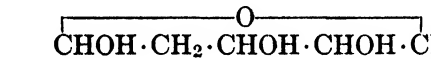
[82] *Quart. J. Exptl. Physiol.*, **7**, 87 (1913).
[83] See for example the data of P. Hodgson and H. B. Lewis, *Am. J. Physiol.*, **87**, 288 (1928).
[84] *J. Biol. Chem.*, **14**, 9 (1913).
[85] *Ibid.*, **99**, 125 (1932–33).

obtained from the thymus gland (it is also present in fair amount in liver, lymph glands, thyroid, lung, brain, placenta, testicle, fish sperm, etc.). The difference between the two nucleic acids is shown by the products which they yield on hydrolysis.[86]

PRODUCTS OF HYDROLYSIS OF NUCLEIC ACIDS

Thymonucleic Acid	*Yeast nucleic acid*
Phosphoric acid	Phosphoric acid
Adenine	Adenine
Guanine	Guanine
Cystosine	Cystosine
Thymine	Uracil
d-2-Desoxyribose	*d*-Ribose

Similar purines are present in both nucleic acids. One of the two pyrimidines is likewise common to both. The other pyrimidine is uracil in yeast nucleic acid and thymine in thymonucleic acid. The carbohydrate component in yeast nucleic acid is *d*-ribose, whereas, in thymonucleic acid, it is *d*-2-desoxyribose,

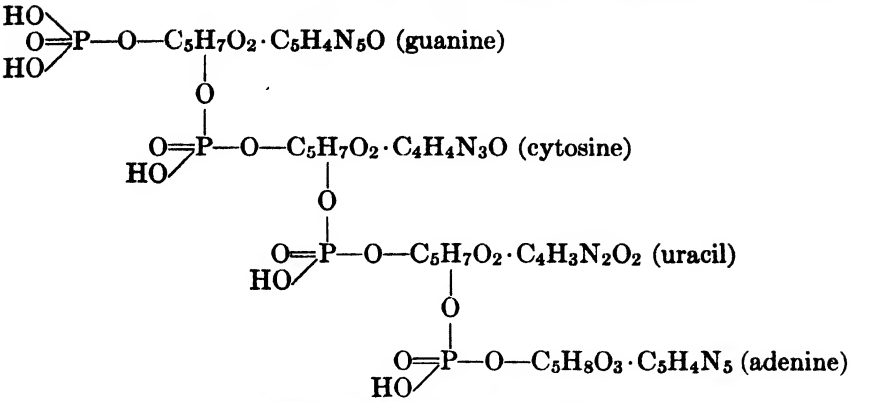


which during the process of hydrolysis is decomposed to levulinic and formic acids.

Concerning the molecular configuration of the nucleic acids, certain details have been fairly well established. The purine or pyrimidine base is apparently united to the sugar, the type of linkage being that of a glucoside. Compounds of this type (sugar—purine or pyrimidine base) are called nucleosides. In turn, each nucleoside is united to a molecule of phosphoric acid (probably by an ester linkage), the point of union being between the sugar and the acid. The phosphoric acid-sugar-base compounds are called nucleotides or mononucleotides. Four such nucleotides constitute a tetranucleotide or nucleic-acid molecule. According to Levene,[87] yeast nucleic acid has the following molecular configuration:

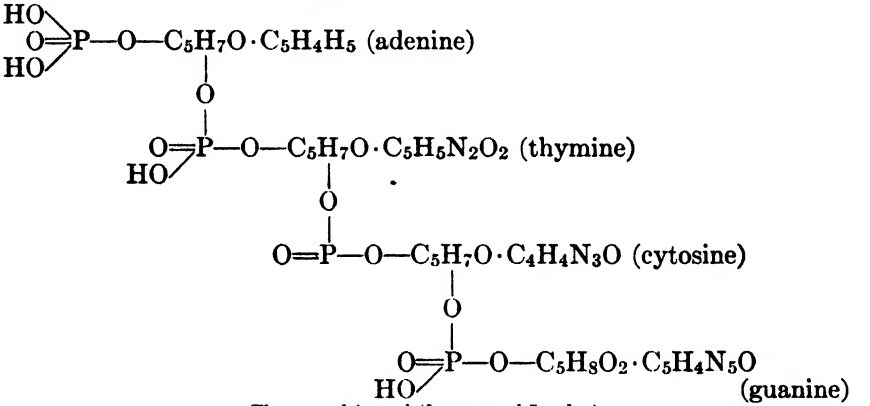
[86] On the basis of this difference a distinction was formerly made between plant and animal nucleic acids, but this distinction has been abandoned since both may coexist in animal tissue. For example, Calvery, on hydrolyzing the β-nucleoprotein prepared from chicken embryos, obtained the same four pentose nucleotides that have been isolated from yeast nucleic acid (*J. Biol. Chem.*, **77**, 489 [1928]).

[87] *J. Biol. Chem.*, **41**, 19 (1920).



Yeast nucleic acid, according to Levene

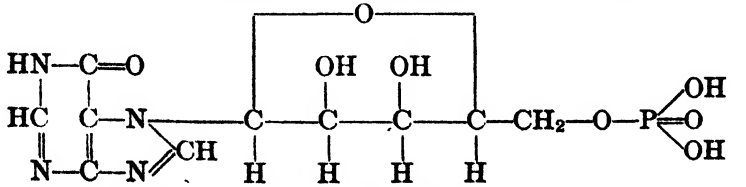
Thymus nucleic acid has a formula analogous to that of yeast nucleic acid (Levene and London).[88]



Thymonucleic acid (Levene and London)

Inosinic, Guanylic, and Adenylic Acids. Three mononucleotides are present as such in animal tissues. Considered in the order of their discovery, these are inosinic, guanylic, and adenylic acids. The first was isolated from meat extract by Liebig; the second was discovered by Hammarsten in pancreatic tissue, and the third in muscle by Embden and Zimmermann.

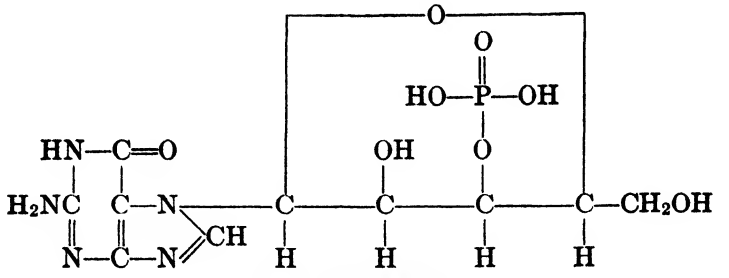
On hydrolysis, inosinic acid yields phosphoric acid, hypoxanthine and *d*-ribose. It is 7′-hypoxanthine-5-phosphoribofuranoside.



Inosinic acid (muscle)

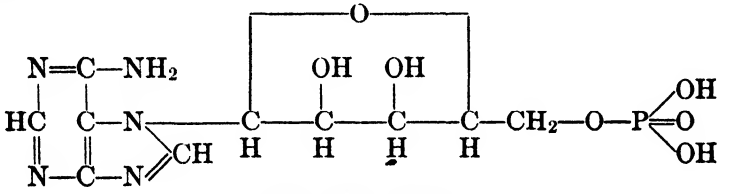
[88] *Ibid.*, **83**, 793 (1929).

Guanylic acid is composed of phosphoric acid, guanine, and *d*-ribose. It is 7-guanine-3-phosphoribofuranoside.



Guanylic acid (pancreas)

The component parts of adenylic acid are: adenine, *d*-ribose, and phosphoric acid. Muscle adenylic acid is 7-adenine-5-phosphoribofuranoside.



Adenylic acid (muscle)

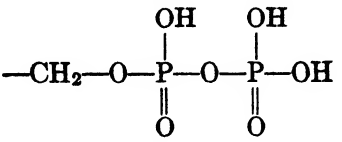
It should be observed that the free nucleotides occurring in muscle, pancreas, etc., contain *d*-ribose. Nucleotides and nucleosides derived from thymonucleic acid by mild hydrolysis have *d*-2-desoxyribose as the constituent sugar.

Nucleotides corresponding to those occurring in muscle may be obtained from yeast nucleic acid. Yeast adenylic acid (7-adenine-3-phosphoribofuranoside) and yeast guanylic acid (7-guanine-3-phosphoribofuranoside are derived by gentle hydrolysis of yeast nucleic acid. Deamination of yeast adenylic acid yields yeast inosinic acid (7-hypoxanthine-3-phosphoribofuranoside); deamination of yeast guanylic yields yeast xanthylic acid (7-xanthine-3-phosphoribofuranoside). It is seen therefore that adenylic and inosinic acids derived from yeast differ from the corresponding nucleotides of muscle. Guanylic acid of pancreas (spleen and liver) is apparently identical with the guanylic acid derived from yeast nucleic acid.

Mention has been made elsewhere of the occurrence of adenosinetriphosphoric acid (adenylpyrophosphoric acid) and of its importance in carbohydrate metabolism (fermentation by yeast, muscle contraction, and lactic acid production). To recapitulate, adenylic acid is capable of absorbing phosphate from phosphate donators (phosphopyruvic acid, phosphocreatine, and hexosediphosphate), while in turn adenyltriphos-

phoric acid thus formed is capable of giving up its more labile phosphate groups for the purpose of esterifying carbohydrate, creatine, etc. (p. 329).

Heart muscle contains adenosinediphosphoric acid.[89] It has essentially the same structure as adenylic acid, the second phosphoric acid group being attached as indicated by the following partial formula:



Another substance related to the nucleotides that is of extraordinary interest is the coenzyme of Warburg and Christian.[90] It occurs both in muscle and yeast and consists of one molecule of adenine, one molecule of nicotinic acid amide, two molecules of pentose, and three molecules of phosphoric acid. The function of this coenzyme as a hydrogen transporter and its participation in the oxidation processes of yeast fermentation have been discussed. A second substance differing only in that it contains two instead of three molecules of phosphoric acid has been designated by Euler as "cohydrase II." It, and Warburg and Christian's coenzyme (codehydrase I of Euler) are the components of yeast cozymase, according to Euler and co-workers.[91]

Digestion of Nucleoproteins and Nucleic Acids. The nucleoproteins of the food are converted by the proteolytic enzymes of the gastric and pancreatic secretions into protein and nucleic acid. Hydrolysis of nucleic acid to mononucleotides is brought about by a specific enzyme found in the intestinal mucosa and juice and designated *polynucleotidase* (Levene and Dillon[92]). Because of the specificity of this enzyme for thymonucleic acid, it has also been described as *thymonuclease* (Klein[93]). A second enzyme acts on nucleotides, removing the phosphate group and yielding nucleosides. This enzyme is non-specific and hydrolyzes other phosphoric acid esters (hexosephosphate, glycerophosphate); hence its designation as a *phosphatase*.

It is assumed that nucleosides may be absorbed without further hydrolysis. However, the intestinal mucosa of at least certain animals contains a *nucleosidase* specific for purine nucleosides.[94] Adenosine (ade-

[89] K. Lohmann, *Biochem. Z.*, **282**, 106 (1935); J. K. Parnas and C. Lutwak-Mann, *ibid.*, **278**, 11 (1935).

[90] *Ibid.*, **275**, 112; **275**, 464 (1935); O. Warburg, W. Christian, and A. Griese, *ibid.*, **282**, 157 (1935).

[91] *Z. physiol. Chem.*, **238**, 233; **240**, 113 (1936).

[92] *J. Biol. Chem.*, **96**, 461 (1932).

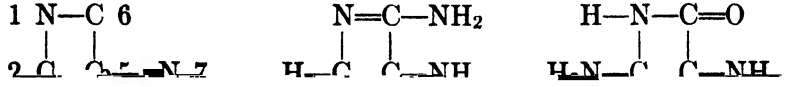
[93] *Z. physiol. Chem.*, **207**, 125 (1932); F. Bielschowsky and W. Klein, *ibid.*, 202 (1932).

[94] The nucleosides are named after the purine or pyrimidine base which they contain. Thus the nucleoside of guanine is guanosine; of adenine, adenosine; of cytosine, cytidine; and of uracil, uridine.

ninedesoxyriboside) is deaminized by an enzyme of the intestinal mucosa.

Aside from their occurrence in intestinal mucosa, polynucleotidases, nucleotidases (phosphatases), and nucleosidases have been prepared from other organs and tissues (spleen, lung, lymph glands, red bone marrow, liver, thymus, kidney, testes, heart, skeletal muscle, brain, etc.). Klein[95] has submitted evidence that purine and pyrimidine nucleosidases coexist in some tissues. He has succeeded in separating the two and in demonstrating that kidney contains relatively more of the pyrimidine nucleosidase than either spleen or bone marrow.

Chemistry of the Purines and Pyrimidines. The structural relationships of the purines and pyrimidines that are of special interest in metabolism are indicated below.



and spinach contain appreciable amounts. Smaller quantities are present, likewise, in wheat, rye, and other grains.

However, as the body is able to synthesize nucleic acid readily, it is doubtful whether it has to depend on the exogenous supply for its anabolic needs. One of the best examples of nucleic-acid synthesis in the animal body is that first observed by Miescher,[97] who showed that the salmon, during its long migration from the sea to its spawning grounds, though abstaining from food, forms large amounts of nuclear material from its own tissues. Likewise, during the incubation of an egg, there is a progressive increase in the content of purine bases (Mendel and Leavenworth).[98] It may be noted here that there is a similar formation of creatine during incubation.

Further evidence of purine synthesis is to be found in the experiments of Burian and Schur,[99] who compared the purine content of newborn rabbits and puppies with the concentrations in the tissues after varying periods of growth on a diet limited to the milk of the mother. Much greater increases were found than could be accounted for on the basis of the purine content of the milk consumed during the periods of the experiments. Somewhat similar were the observations of Kollmann,[100] who showed that human individuals, though kept on a very low purine diet, not only gained weight, but eliminated much larger quantities of uric acid than could be accounted for from the purine intake.

Benedict [101] has demonstrated the synthesis of purines in the Dalmatian coach hound. In this species of dog, as in man, the end-product of purine metabolism is uric acid and not allantoin as in other dogs. Benedict kept a Dalmatian dog on a purine-free diet, and yet this animal continued to excrete uric acid.

The conversion of histidine into purines has been discussed elsewhere (p. 403). Of other non-purine precursors there is no definite knowledge.

Metabolism of Adenine and Guanine; Origin of Uric Acid. Endogenous nuclear metabolism probably involves the preliminary hydrolysis of nucleic acids through the same stages as in digestion. It may also be assumed that the nucleosides, purines, and pyrimidines derived from the tissue nucleoproteins and those derived from the food share the same fate in metabolism.

The fact that adenosine occurs in the urine (Calvery)[102] indicates that a portion of the nucleoside escapes further change.[103] The rest on hydrol-

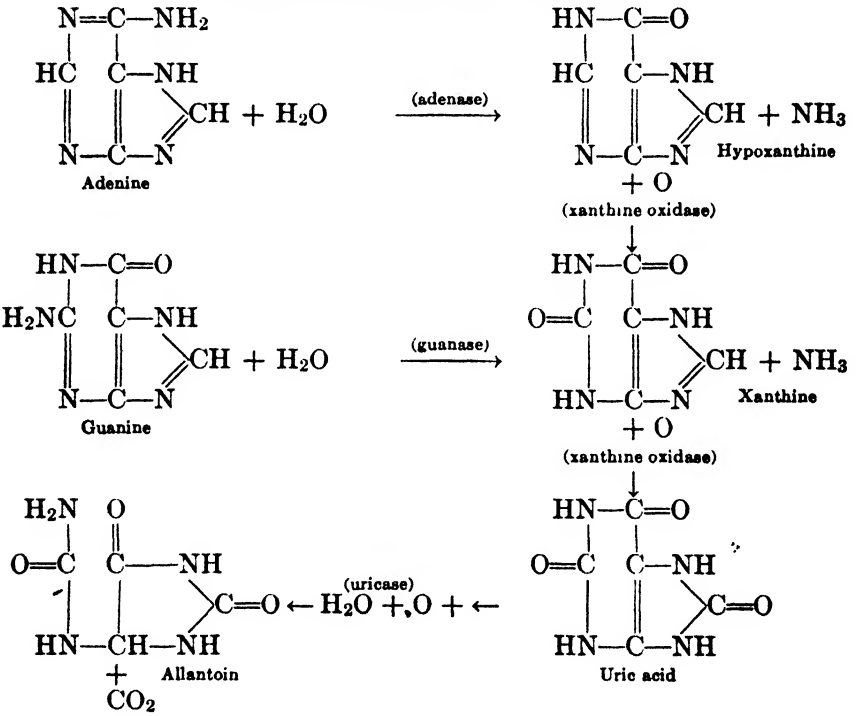
[97] Cited by Rose,[96] p. 555.
[98] *Am. J. Physiol.*, **21**, 77 (1908).
[99] *Z. physiol. Chem.*, **23**, 55 (1897).
[100] *Biochem. Z.*, **123**, 235 (1921).
[101] *J. Lab. Clin. Med.*, **2**, 1 (1916); *Harvey Lectures*, 1915–16, p. 346.
[102] *J. Biol. Chem.*, **86**, 263 (1930).
[103] In a study of the fate of the purine nucleosides in the dog, Allen and Cerecedo, *J. Biol. Chem.*, **102**, 313 (1933); **107**, 421 (1934), found that when fed in moderate amounts (2–3 grams) guanosine and adenosine were completely metabolized. About half of the guanosine nitrogen appeared as allantoin and one-third as urea. Adeno-

ysis yields adenine, or it may form inosine by deamination. Adenine is converted by *adenase* into hypoxanthine, whereas the same compound is formed from inosine by the action of nucleosidase. Hypoxanthine is converted into xanthine by *xanthine oxidase*, which is a dehydrogenase.

Similarly guanosine on hydrolysis yields guanine, or, by deamination, it may be converted into xanthosine. Guanine, by the action of *guanase*, is changed to xanthine, whereas a nucleosidase acting on xanthosine would yield the same product. In man, xanthine is finally converted into uric acid. These transformations and relations are outlined below.

Adenosine → Inosine Guanosine → Xanthosine
↓ ↓ ↓ ↓
Adenine → Hypoxanthine → Xanthine Guanine → Xanthine

FATE OF ADENINE AND GUANINE



Allantoin.[104] In the Mammalia, excepting man, the anthropoid apes,

sine yielded a similar proportion of allantoin, but there was no increase in urea nitrogen. From these observations it was concluded that purine metabolism in the dog does not end with allantoin, but that a portion of this is metabolized further, one of the products being urea. Guanine when fed to dogs in small amounts is partly converted into allantoin and partly into urea.

[104] The name is derived from the fact that it was first encountered in allantoic fluid (cow).

and the pure-bred Dalmatian coach hound, allantoin is the chief end-product of purine metabolism, its formation from uric acid being indicated above.

The conversion of uric acid into allantoin is attributed to the action of the enzyme *uricase*, which is evidently lacking in man, the higher apes, and the Dalmatian dog.[105, 106]

Bollman, Mann, and Magath [107] have shown that the destruction of uric acid and its conversion into allantoin in the dog is dependent on the liver, since complete extirpation of this organ results in the excretion of uric acid.

In the amphibia and the elasmobranchs, urea is the end-product of both nitrogen and purine metabolism. These organisms have, in addition to liver arginase, the ornithine cycle (p. 411), xanthine oxidase and uricase, two enzymes, *allantoinase*, which converts allantoin into allantoic acid, and *allantoicase*, which turns allantoic acid into urea and glyoxylic acid. The teleosts lack the ornithine cycle, but have all the other enzymes (Baldwin).[108] Accordingly urea is the end-product of purine metabolism and ammonia is the end-product of protein metabolism.

Purine Metabolism in Birds and Reptiles. In birds and reptiles uric acid is not only the end-product of purine metabolism, but also the principal end-product of protein metabolism. How the uric acid is formed from non-purine precursors is not known definitely. It has been supposed that urea was the first product, reacting with tartronic acid (COOH·CHOH·COOH) to yield dialuric acid, which in turn combined with another molecule of urea to form uric acid.

This theory, advanced by Wiener,[109] is obviously untenable in view of the absence from bird and reptile liver of arginase, the enzyme that is apparently essential in the formation of urea. Moreover, perfusion experiments using bird liver, as well as studies with tissue slices, have failed to demonstrate the production of uric acid from urea and the other alleged precursors.

[105] The presence of allantoin in human urine, especially in pregnant women and nurslings, was claimed by some of the earlier investigators. Employing a recently developed technique, Larson, *J. Biol. Chem.*, **94**, 727 (1931–32), found the daily excretion in man to be about 25–30 mg. This is a small fraction of the average uric acid excretion, and although it is conceivable that it represents a further stage in purine metabolism than uric acid, it is perhaps just as likely that it is exogenous in origin, representing the allantoin ingested with the food.

[106] Among recent contributions pertaining to the action and properties of uricase are the following: R. Truszkowski, *Biochem. J.*, **24**, 1340, 1349, 1359 (1930); **26**, 285 (1932); M. Z. Grynberg, *Biochem. Z.*, **236**, 138 (1931); W. Schuler, *Z. physiol. Chem.*, **208**, 237 (1932); K. Ro, *J. Biochem.* (*Japan*), **14**, 361 (1931–32).

[107] *Am. J. Physiol.*, **72**, 629 (1925); J. L. Bollman and F. C. Mann, *ibid.*, **104**, 242 (1933).

[108] E. Baldwin, "An Introduction to Comparative Biochemistry," Macmillan, New York, 1936.

[109] *Beitr. chem Physiol. Pathol.*, **2**, 42 (1902).

In a series of experiments Schuler and Reindel [110] have attempted to elucidate the problem of uric-acid production. Employing liver slices (pigeon), they have succeeded in demonstrating the utilization of amino acids as the source of nitrogen and the formation of xanthine, but they have failed so far to establish the nature of the intermediates. Edson and co-workers [111] in duplicating these experiments concluded that hypoxanthine was the first purine formed.

Excretion of Uric Acid. Uric acid is excreted in combination with sodium, potassium, and ammonium in the form of urates. In addition to uric acid, there are probably smaller amounts of other purines in the urine, such as adenine, xanthine, hypoxanthine, and methylpurine derivatives. The daily excretion of uric acid is subject to considerable variation, being influenced by diet and other factors.[112] On a purine-free diet, the normal daily excretion is between 0.2 and 0.4 gram. The content of uric acid in the blood usually varies between 1 and 3 mg. per 100 cc. Marked retention of uric acid occurs in nephritis, in gout, and, as shown by Lennox,[113] during starvation. According to Lennox, uric acid retention is in some way associated with ketosis. In conditions such as leukemia and pneumonia, which are associated with marked destruction of nuclear material, the uric-acid content of the blood and urine increases appreciably.

Strenuous muscular work causes an increase in the uric acid concentration of the blood, accompanied by diminished excretion in the urine. Quick [114] has offered the explanation that the retention of uric acid is probably related to overproduction of lactic acid, since the same effect is obtained by feeding the latter.

In this connection it is interesting to note that Kerr,[115] working in Embden's laboratory, demonstrated a marked increase in the purine fraction of isolated frog muscle stimulated to the point of exhaustion.

The glucose concentration of the blood seems to be another factor controlling the amount of blood uric acid. Chaikoff and Larson [116] have observed that insulin hypoglycemia in the Dalmatian dog causes a rise in blood uric acid and also an increase in output. Since epinephrine produced the same effect, it has been surmised that the influence of insulin hypoglycemia on purine metabolism is more directly related to the epinephrine secreted in response to the hypoglycemia.

Coffee, tea, and cocoa contain methylpurine derivatives, as well as

[110] *Z. physiol. Chem.*, **221**, 232 (1933); **234**, 63 (1935); **247**, 172; **248**, 197 (1937).

[111] N. L. Edson, H. A. Krebs, and A. Model, *Biochem. J.*, **30**, 1380 (1936).

[112] For a detailed discussion of the uric acid problem and the factors influencing its excretion see Folin, O., Berglund, H., and Derick, C., *J. Biol. Chem.*, **60**, 361 (1924).

[113] *J. Biol. Chem.*, **66**, 521 (1925).

[114] *J. Biol. Chem.*, **110**, 107 (1935).

[115] *Z. physiol. Chem.*, **210**, 181 (1932).

[116] *J. Biol. Chem.*, **109**, 85, 395 (1935).

amino- and oxypurines, tea being especially rich in adenine. Calvery [117] has prepared both guanine nucleotide and cytosine nucleotide from dried tea leaves and is of the opinion that a pentose nucleic acid is a natural product of tea. It is unlikely that all the methylated purines are converted into uric acid in metabolism. Some of these are probably excreted partly unchanged and partly after demethylation to monomethylpurine. Caffeine and theophylline, after ingestion, increase the elimination of uric acid.[118] Theobromine, on the other hand, does not seem to be converted into uric acid.

Metabolism of the Pyrimidines. Our knowledge of the fate of the pyrimidines in metabolism is less complete than that of the purines. Sweet and Levene [119] found that, if they fed thymine to a dog, more than half could be recovered in the urine, but if the same amount of thymine was fed in the form of nucleic acid, none could be recovered in the urine. D. W. Wilson [120] performed similar experiments with uracil in rabbits and on a human subject. When administered as such, uracil was quantitatively excreted unchanged, but with uracil combined in the form of a nucleoside or nucleotide, very little of the uracil could be found in the urine. Nearly all of it had apparently undergone metabolism, the end-product of which was urea. On the basis of these and similar observations, Wilson suggested that the intermediary metabolism of nucleic acids involves radical changes in both the purine and pyrimidine groups before the relatively complex combinations (nucleosides and nucleotides) are broken up.

Deuel [121] observed that when large quantities of thymine or uracil (1–3 grams) were given to dogs a considerable proportion appeared in the urine. However, when the same amounts were given in small divided doses over a period of days, the pyrimidines were apparently metabolized, for none could be detected in the urine. When a large amount (50 grams) of thymus nucleic acid was fed, the urine was found to contain free pyrimidines. Apparently, a sufficient amount of free pyrimidine had been liberated so that a portion escaped oxidation. This does not occur, under normal conditions, according to Deuel, who found it impossible to isolate even a trace of pyrimidine in 150 liters of human urine.

Results similar to those of Deuel were also obtained by Cerecedo.[122] When fed in small amounts to dogs, uracil and thymine were metabolized, the predominant end-product being urea. Cytosine, on the

[117] *Ibid.*, **72**, 549 (1927).

[118] L. B. Mendel and E. L. Wardell, *J. Am. Med. Assoc.*, **68**, 1805 (1917); V. C. Myers and Wardell, *J. Biol. Chem.*, **77**, 697 (1928); see also R. F. Hanzal and V. C. Myers, *ibid.*, **97**, Proc. lxix (1932).

[119] *J. Exptl. Med.*, **9**, 229 (1907).

[120] *J. Biol. Chem.*, **56**, 215 (1923).

[121] *Ibid.*, **60**, 749 (1924).

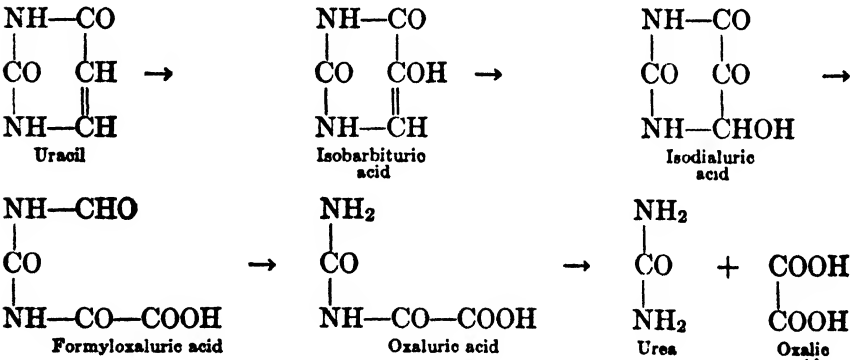
[122] *Ibid.*, **75**, 661 (1927); **87**, 453 (1930); *Proc. Soc. Exptl. Biol. Med.*, **27**, 203 (1929).

other hand, escaped oxidation, being partly excreted unchanged and partly deaminized to uracil. In a later experiment, Emerson and Cerecedo confirmed the observation that cytosine, administered as such, is not utilized directly, but that when given in combination as the nucleoside, is completely metabolized.

When the observations just recorded are summarized, the following conclusions seem justified: (1) uracil and thymine when present in small amount as the free base are readily utilized, (2) cytosine as such is not utilized directly, being partly excreted unchanged and partly converted into uracil, (3) pyrimidines in nucleoside combination, particularly cytosine nucleoside, are more readily and more completely metabolized than the free pyrimidines, (4) normally the urine contains little, if any, pyrimidines; possibly when large quantities of nucleoproteins are fed, the urine may contain a small amount of unchanged pyrimidines. The constituent pentose of the nucleosides appears to be completely utilized.

The metabolism of one of the pyrimidines, uracil, has been thoroughly investigated by Cerecedo.[123] Inasmuch as the *in vitro* oxidation of uracil yields isobarbituric acid and in turn isodialuric acid, these compounds were fed to dogs in small amounts. It was found that to a considerable extent they were metabolized to urea.

Isobarbituric acid is oxidized by potassium permanganate successively to formyloxaluric and oxaluric acids. When these compounds were fed to dogs they were likewise partly catabolized to urea. It was therefore assumed that the metabolism of uracil involves the following sequence of reactions:

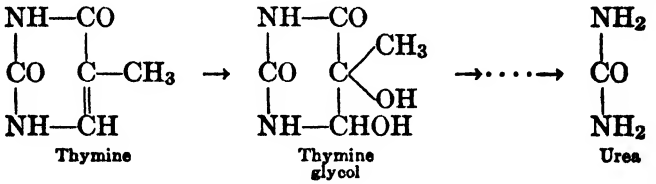


It was noted that, after the administration of isobarbituric, isodialuric, or formyloxaluric acid, the urinary output of inorganic sulfates diminished, while the ethereal sulfates increased correspondingly, suggesting that these derivatives of uracil were partly excreted as conjugated sulfates. This has been confirmed by Stekol and Cerecedo [123] in

[123] *J. Biol. Chem.*, **88**, 695 (1930); **93**, 269, 283 (1931); J. A. Stekol and L. R. Cerecedo, *ibid.*, **93**, 275 (1931); **100**, 653 (1933).

a recent experiment on human subjects. When isobarbituric acid was fed, it was partly catabolized to urea and partly excreted as an ethereal sulfate, the latter being formed at the expense of the inorganic sulfur fraction.

Less is known of the metabolism of thymine. According to Cerecedo, thymine glycol is an intermediate product in its conversion to urea.



The partial conversion of cytosine, when fed as such, into uracil has been mentioned. Whether this represents an intermediate stage in the metabolism of cytidine, the corresponding nucleoside, remains to be determined.

SULFUR METABOLISM

The main features of sulfur metabolism were discussed in the preceding chapter in connection with the fate of cystine and methionine in the body. These amino acids seem to be the principal sources of sulfur in metabolism and it has been established that methionine is essential for maintenance and growth. A small amount of sulfur is probably derived from the sulfur-containing lipids or sulfatides of the diet. Inorganic sulfates are apparently of no importance from the standpoint of nutrition.

Among the more familiar physiological constituents, other than protein, that contain sulfur, may be mentioned glutathione, taurine, insulin, and the sulfolipids contained in the tissues of the central nervous system and in the secretions of the skin. Sulfur is removed from the body in several ways. In the growth of hair, considerable quantities are lost in the form of cystine, which is especially abundant in the albuminoid, keratin. The amount of methionine in keratin is comparatively small, representing less than 5 per cent of the total sulfur. The bile contains sulfur compounds which, if not reabsorbed, are excreted in the stools. The saliva, urine, and bile contain thiocyanates, probably formed in the detoxication of small amounts of CN arising in metabolism. The urine is the most important channel of excretion of the end-products of protein metabolism, including those containing sulfur. These have been referred to previously and will receive further attention in the next chapter.

The Ratio of Nitrogen to Sulfur in the Urine. A considerable amount of work has been done in studying the ratio of the excretion of sulfur to the excretion of nitrogen with a view to elucidating certain problems in protein metabolism. The results obtained by one group of

workers (von Wendt,[124] etc.) have led them to believe that, after the ingestion of protein, the sulfur is excreted earlier than the nitrogen. If this were true, it would mean that the protein which the body retains is poorer in sulfur than the original protein of the diet.

In contrast to this, others (Gruber,[125] etc.) have found that the N : S ratio is identical with that of the protein fed. Then there are the observations of Lewis [126] and of Fay and Mendel,[127] who have shown a frequent and apparently specific retention of sulfur after periods of starvation. This is highly suggestive, for it indicates the organism may be capable of storing protein of varying composition in response to specific needs, a concept which has considerable additional evidence in its favor (Wilson).[128] For a summary of the recent literature, the student is referred to Lewis' excellent reviews [129] on sulfur metabolism.

As to the numerical value of the starvation N : S ratio, von Wendt determined it to be about 9.3, but nearly all other investigators have obtained values ranging between 13 and 16 with an average of about 14. This means that for every gram of sulfur normally excreted in the urine, the nitrogen excretion is about 14 grams. These values correspond rather well to the proportions of sulfur and nitrogen in muscle proteins.

Summary. Some of the main features of protein and nucleic-acid metabolism are summarized in the diagram given on p. 443.

[124] *Skand. Arch. Physiol.*, **17**, 211 (1905).

[125] *Z. Biol.*, **42**, 407 (1901); compare with S. Morgulis, *J. Biol. Chem.*, **77**, 627 (1928).

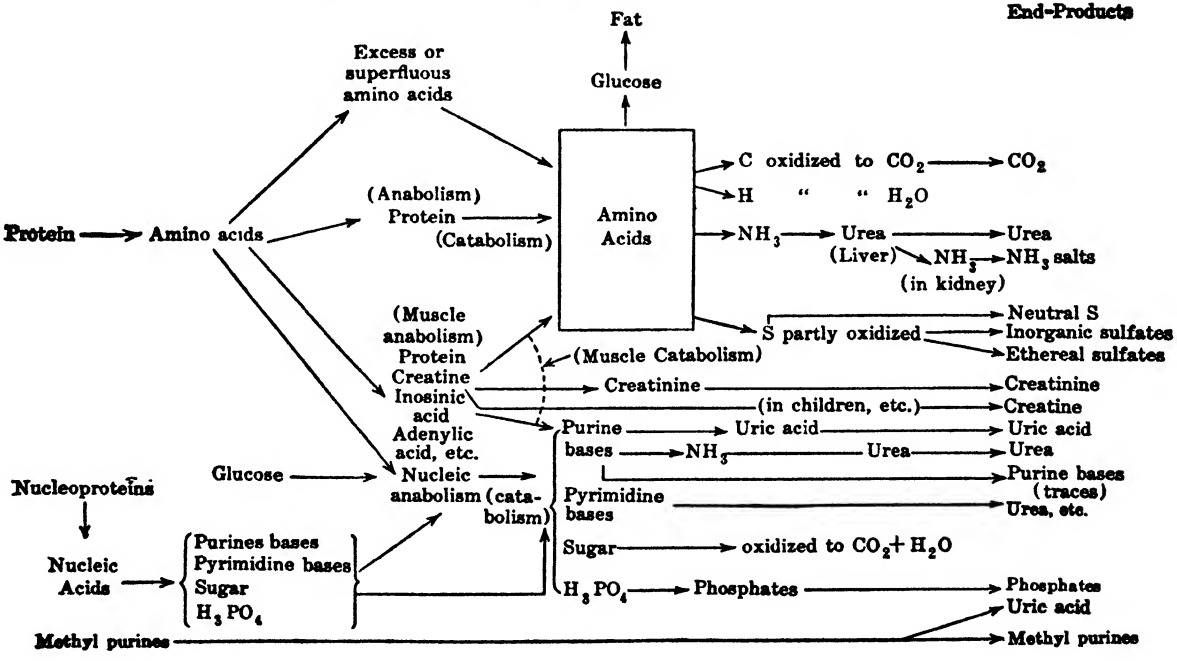
[126] *J. Biol. Chem.*, **26**, 61 (1916).

[127] *Am. J. Physiol.*, **75**, 308 (1926).

[128] *Biochem. J.*, **19**, 322 (1925); **20**, 76 (1926); *J. Physiol.*, **72**, 327 (1931); **77**, 240 (1933). See also E. P. Cathcart, "The Physiology of Protein Metabolism," 1921 edition, pp. 105, 123.

[129] *Physiol. Rev.*, **4**, 394 (1924); *Ann. Rev. Biochem.*, **1**, 171 (1932); **2**, 95 (1933); **4**, 149 (1935); see also V. du Vigneaud and H. M. Dyer, *ibid.*, **6**, 193 (1937).

SUMMARY OF PROTEIN AND NUCLEIC-ACID METABOLISM



CHAPTER XV

EXCRETION: THE URINE

Channels of Excretion. The removal of waste products from the body is accomplished largely, but not solely, by the lungs and kidneys. The former are concerned mainly with the excretion of gaseous waste products, the latter with the elimination of solids in solution. Among the other organs which may be regarded as at least in part excretory are the liver and the gall bladder. These are concerned with the removal of cholesterol, bile pigments, and other substances. The intestinal epithelium excretes inorganic constituents, especially those foreign to the body. The saliva contains small quantities of nitrogenous and other waste products. Not only water, but also compounds such as urea, uric acid, lactic acid, and sodium chloride are excreted by way of the skin. The daily output of perspiration contains about 0.1–0.3 gram of nitrogen.[1] A very small amount of nitrogen is lost to the body daily in the growth of hair and nails. In careful studies of nitrogen balance, it is desirable to take into account all the channels of excretion, so as to allow for all losses. Due allowance must also be made for the excretion of nitrogenous material by the intestinal epithelium, as this usually amounts, even in starvation, to about 0.5 gram per day. Thus the extra-renal excretion of nitrogen may be estimated at about 1.0 gram.

Formation of Urine. Textbooks of physiology and histology contain descriptions of the kidney. For a proper understanding of what is to follow, the student should be familiar with the microscopic anatomy of this organ.

Various theories have been proposed to explain kidney secretion. According to the theory advanced by Ludwig in 1844, the glomeruli filter from the blood its non-colloidal constituents. Large amounts of fluid thus pass into the tubules. During its passage through the tubules, the fluid becomes concentrated by the reabsorption of water.

According to the theory put forward by Bowman and later extended by Heidenhain, water and salts are secreted by the glomerulus, whereas the organic constituents are added to the urine as a result of the secretory activity of the renal tubular epithelium.

[1] G. A. Talbert and his associates have published a series of papers on the composition of sweat: *Am. J. Physiol.*, **81**, 74 (1927); **82**, 153, 639 (1927); **84**, 577 (1928); **97**, 426 (1931); **100**, 328 (1932); see also H. H. Mosher, *J. Biol. Chem.*, **99**, 781 (1932–33); E. H. Fishberg and W. Bierman, *ibid.*, **97**, 433 (1932).

Cushny [2] in his monograph, " The Secretion of the Urine," reviewed the evidence for and against these theories, and advanced what he termed the " modern theory," according to which water and the non-colloidal constituents of the plasma are filtered through the glomerulus. He assumed that, as the glomerular filtrate proceeds through the tubules, only those substances are reabsorbed that are necessary to the plasma and tissues. Such substances are water, glucose, salts, amino acids, sodium bicarbonate, etc., and are described as " threshold " substances. There is relatively less or no absorption of other constituents, such as sulfate, phosphate, urea, and creatinine. These are designated " low-threshold " or " non-threshold " substances.

Experiments conducted in Richards' laboratory [3] have materially strengthened the view that the glomerular urine is an ultrafiltrate of the plasma. By means of ingenious technical procedures and adaptations of available microanalytical methods, Richards and associates have been able to remove and analyze the fluid secreted by single glomeruli (frog), as well as the contents of different segments of individual tubules (frog, *Necturus*). In this way it has been possible to show that water and glucose are reabsorbed chiefly from the proximal end, while chloride is reabsorbed mainly from the distal portion. The urine also becomes more acid toward the distal end, presumably because of reabsorption of bicarbonate. Substances not absorbed, such as urea, uric acid, creatinine, and phosphate, increase in concentration in the tubules. In most instances the increases in concentration were found to correspond to the water reabsorbed, but in the frog the concentration of urea was proportionately higher than could be accounted for on this basis, suggesting tubular secretion of urea. It is, of course, not known precisely to what extent these relations apply to the mammalian kidney.

Blood pressure and the volume of blood flow through the kidney influence the amount of urine formation.[3a] Richards and Plant [4] perfused the rabbit's kidney with hirudinized blood in a manner which permitted of variations in the pressure within the renal circulation without alterations in the rate of blood flow. It was found that changes

[2] A. R. Cushny, "The Secretion of the Urine," Longmans, Green & Co., New York, 1917.

[3] A. N. Richards, "Urine Formation in the Amphibian Kidney," *Harvey Lectures*, **30**, 93 (1934–35).

[3a] The blood pressure in the afferent glomerular arterioles has been estimated to be about 75 mm. Hg, and is therefore considerably higher than the arteriolar pressure in the skin and elsewhere. If the osmotic pressure of the plasma proteins is assumed to be 28 mm. and the pressure in Bowman's capsule 5 mm., the effective glomerular filtration pressure would be 75 − 33 = 42 mm. Blood is delivered to the glomerular vessels under a high head of pressure owing to the fact that the large renal artery (very large in proportion to the bulk of tissue it supplies) breaks up suddenly into short branches. Another factor is the disparity between the diameters of the afferent and efferent glomerular vessels. For a detailed account, consult C. H. Best and N. B. Taylor, "Physiological Basis of Medical Practice," 1937, Chapter 36.

[4] *Am. J. Physiol.*, **59**, 144 (1922).

in renal blood pressure produced parallel changes in the rate of urine flow.

The rate with which certain substances, such as urea and phosphates, are excreted by the kidneys seems to be regulated not only by their concentration in the blood but by a variety of other factors. For details the student is referred to the review of Marshall, the papers of Addis and his associates,[5] and to the discussion of urea clearance.

It is instructive to compare the concentration of various constituents in the blood and urine. The values given here, though in no sense fixed, are nevertheless fairly representative. Urine contains roughly 25 times as much uric acid, 40 times as much ammonia, 60 times as much urea, 100 times as much creatinine, 30 times as much PO_4, and 60 times as much SO_4, as an equivalent volume of blood plasma contains. On the other hand, there is little difference in the concentration of chloride, sodium, calcium, and magnesium; and much less glucose is present in the urine than in the blood. On the basis of Cushny's theory, this would mean the filtration of large quantities of plasma per day. Basing his computations on the difference in the concentrations of urea, Cushny calculated that, in the formation of 1 liter of urine, 67 liters of plasma would have to filter through the glomeruli.

This must entail a considerable amount of work, the evaluation of which has been attempted by several investigators, notably by Borsook and Winegarden,[6] who approached the problem from the standpoint of thermodynamics. Their study indicates that the excretion of a urine isotonic with the plasma would require no work on the part of the kidney. Ordinarily the work *performed* by the kidney in man is of the order of magnitude of 0.7 gram-calorie per cubic centimeter of urine, or 70 gram-calories per gram of nitrogen excreted. However, this is a very small fraction of the energy *consumed* by the kidneys. This has been estimated to be 6–11 kilogram-calories per gram of nitrogen, showing that although the normal kidney has a great capacity for work, the " efficiency " with which it is performed is probably not greater than 1–2 per cent. In disease of the kidney, its capacity for work is markedly reduced.

Physical Properties; *Volume.* The volume of urine secreted per day (twenty-four hours) may vary within wide limits. A normal adult usually excretes 1200–2000 cc. The most important factor determining the output of urine is obviously the water intake, in the form of water, milk, soup, or beverages. Temperature is another important modifying factor. During the summer months or in warm climate, less urine is formed, because of the loss of water in perspiration. Urination is, as a rule, more frequent in winter than in summer. A high-protein diet, by giving rise to nitrogenous end-products having a diuretic effect,

[5] E. K. Marshall, *Physiol. Rev.*, **6**, 440 (1926); Addis, Barnett, and Shevky, *Am. J. Physiol.*, **46**, 1, 39, 52 (1918); Addis and Drury, *J. Biol. Chem.*, **55**, 105, 629, 639 (1923); Addis, Meyers, and Bayer, *Am. J. Physiol.*, **72**, 125 (1925).

[6] *Proc. Natl. Acad. Sci. U. S.*, **17**, 3, 13 (1931).

causes an increased elimination of urine. Muscular exercise, on the contrary, results in a diminished volume of urine. Nervousness and excitement may cause abnormally frequent and abundant micturition. It is generally believed that mentally deranged people excrete more urine than normal individuals. The diuretic effect of coffee, tea, and chocolate is due largely to the presence of caffeine and other purine derivatives.

Normally, the amount of urine secreted varies with the time of day. For an hour or two after a meal, there is usually an increase in urine formation. During the night much less urine is formed than during the day. If the total urine for twenty-four hours is 1500 cc., that formed during the night is usually about 400 or 500 cc. Simpson has observed that these relations hold even when a definite amount of water is given at hourly intervals during the day and night. Apparently there is a retention of water during the night, whereas during part of the day there is a negative water balance. These variations seem to be dependent, at least in part, on body temperature. Coincident with the rise in body temperature which occurs at about 6 A.M., there is an increased secretion of urine. Both the temperature and the urine volume continue to increase until late in the afternoon or evening, after which both begin to fall until the following morning when the cycle begins again.

The chloride and phosphate elimination and *p*H of the urine are likewise decreased during sleep, but it is interesting to note that these changes may occur irrespective of significant changes in the urine volume (Simpson).[7]

In nephritis, the urine collected during short intervals of the day and night shows less than the normal variation in volume, specific gravity, and composition. As a rule, the night urine of nephritics is proportionately more abundant than that of normal individuals. The condition in which an excessive amount of urine is secreted at night is called nocturia.

The determination of urine volume may be of value in diagnosing kidney disease. In acute nephritis due to mercuric chloride, there may be complete suppression of urine, or anuria. Oliguria is the condition of low urine output and is observed in eclampsia, heart failure, fever, and diarrhea. Polyuria, or excessive secretion of urine, occurs especially in diabetes mellitus and diabetes insipidus. In diabetes insipidus, the daily elimination of urine may exceed 20 liters, and there is at least one case on record in which 50 liters were excreted during a period of twenty-four hours.

Color. Normal urine is pale yellow in color, but may vary from a slight yellow tinge to deep amber-yellow, depending on its concentration. In fever, the urine is usually dark yellow or brown-red in color. In

[7] *J. Biol. Chem.*, **59**, 107 (1924); **67**, 505 (1926); **84**, 393 (1929); compare N. Kleitman, *Am. J. Physiol.*, **74**, 225 (1925).

jaundice, the presence of bile pigments gives the urine a greenish yellow or greenish brown color. The presence of blood or hemoglobin would obviously cause a reddish tinge. Brown and black urine may be due to the presence of methemoglobin, melanin, and phenol derivatives such as are excreted in carbolic-acid poisoning. Drugs excreted in the urine may likewise give rise to peculiar colors.

Drabkin [8] has made the important observation that normally the output of urinary pigment is constant from day to day and is independent of the diet. It is accordingly a product of endogenous metabolism, being eliminated in quantities which are proportional to its intensity. Experimentally the output of pigment was increased by fasting, the administration of acids, or the administration of calorigenic agents such as epinephrine or thyroxine. A diminished urinary pigment output was observed after the administration of alkali, or following the surgical removal of the thyroid gland. In exophthalmic goiter, the quantity of pigment is abnormally high. In one case which was followed daily, the amount of urinary pigment paralleled the patient's metabolic rate.

A pigment, uropterine, occurs in urine which seems to be identical with xanthopterine found in the wings of the brimstone butterfly (*Gonopteryx rhamni*).[9]

Transparency. Freshly voided urine is usually clear and transparent, except after a hearty meal, when the precipitation of calcium phosphate, due to the alkalinity of the urine (alkaline tide), may render it turbid. Strict vegetarians, as well as herbivorous animals, normally excrete an alkaline and turbid urine. Clear urine may become turbid on standing, owing to the precipitation of mucin derived from the urinary tract. The conversion of urea into ammonia by bacteria may cause an acid transparent urine to change into an alkaline turbid urine, the turbidity here being due, likewise, to the precipitation of calcium phosphate.

In the abnormal condition known as chyluria, the urine has a milky appearance, which is due to the presence of fat globules. In inflammations of the urinary tract, large amounts of pus may be excreted with the urine, causing it to acquire a turbid appearance.

Odor and Taste. Urine has a faint aromatic odor which has been attributed to a substance of unknown chemical composition, called urinod. The odor of urine may be influenced by the ingestion of drugs and vegetables. Asparagus causes a peculiar odor, due to methylmercaptan. Abnormal constituents, such as acetone bodies, may modify the odor of urine. Putrefactive changes cause urine to acquire an ammoniacal odor. Normal urine is salty to the taste; diabetic urine has a sweetish taste.

Specific Gravity. The specific gravity of urine depends on its concentration. The greater the volume, the lower is the concentration, and hence the specific gravity. Accordingly, the specific gravity of

[8] *J. Biol. Chem.*, **75**, 443, 481 (1927); **88**, 433, 443 (1930).
[9] W. Koschara, *Z. physiol. Chem.*, **240**, 127 (1936).

normal urine is not fixed but may vary within a wide range of values. The normal range is usually given as 1.008–1.030. A rough estimate of the total solids of the urine, in grams per liter, may be obtained by multiplying the last two figures of the specific gravity (i.e., the second and third decimal places) by the factor 2.66 (Long's coefficient).

Reaction. Whereas the blood is faintly alkaline in reaction (*p*H 7.35–7.43), the urine is normally acid. Indeed, on an ordinary diet, about 250–350 cc. of 0.1 *N* acid is excreted daily. Henderson and Palmer [10] have calculated that the kidneys may normally remove from the body 600–700 cc. of 0.1 *N* acid, and in diabetes the excretion of acid may be ten times as great. It is because of this that the kidneys enable the blood to maintain its reaction within certain narrow limits. When the kidneys fail to function properly, retention of urinary constituents occurs in the blood and is followed by the well-known symptoms of intoxication, which have been incorrectly classed under the term of uremia or uremic poisoning. The coma that characterizes the terminal stages of nephritis is not due so much to the retention of urea and other nitrogenous constituents as to the accumulation of acid. Cushny [11] makes the statement that acid is probably the most poisonous of all the waste products of metabolism known at present.

The kidney exerts its regulatory effect by eliminating acid and at the same time retaining, for the use of the organism, as much base as possible. In the blood the ratio $Na_2HPO_4 : NaH_2PO_4$ is in favor of the basic phosphate, whereas in the urine there is a preponderance of the acid phosphate. The change which brings about this altered relation, and which is believed to take place in the tubule, may be represented as follows:

$$Na_2HPO_4 + H_2O \rightarrow NaH_2PO_4 + Na^+ + OH^-$$

The sodium is retained in combination as bicarbonate. The important point to be emphasized here is this: The glomerular filtrate resembles in composition the blood plasma. Both, therefore, contain Na_2HPO_4 and NaH_2PO_4 in approximately the same proportion. It may be supposed that the acid phosphate is excreted unchanged, although we should not disregard the possibility of a certain amount of conversion into $NH_4H_2PO_4$. The Na_2HPO_4, on the other hand, as it proceeds down the tubule, yields part of its Na, which is reabsorbed by the tubular epithelium.

The shift from basic to acid phosphate and the replacement of fixed base by ammonia in the kidney have been demonstrated by a number of workers. Hendrix and Sanders,[12] for example, observed that the injection of dibasic phosphate caused a marked rise in the titratable acidity and

[10] *J. Biol. Chem.*, **13**, 393 (1913).
[11] A. R. Cushny, "The Secretion of the Urine," p. 165.
[12] *J. Biol. Chem.*, **58**, 503 (1923–24).

ammonia in the urine. In later experiments, Hendrix and Calvin [13] have shown that, in diuresis produced by the injection of certain neutral salts (sodium chloride, sodium nitrate, and sodium sulfate) and urea, there is a loss of base through the kidney over and above that lost normally. The excretion of base is reflected in a marked fall in the alkali reserve of the blood, which occurs simultaneously and which is obviously due to the removal of fixed base from the body. These changes are apparently due to a failure in reabsorption from the tubules, since these are flooded and overtaxed in diuresis, and cannot be supposed to function with their normal efficiency in the retention of basic ions.

Titratable Acidity. A measure of urinary acidity may be obtained by titration. The method in general use is that of Folin, according to which a certain amount of urine (25 cc.), to which 15–20 grams of finely pulverized potassium oxalate have been added, is titrated with standard sodium or potassium hydroxide (0.1 *N*), phenolphthalein being used as the indicator. The end-point is not sharp, because of the presence of ammonium salts. The oxalate is added to precipitate the calcium as oxalate, for otherwise it would interfere with the end-point by prematurely forming insoluble calcium phosphate as the point of neutrality was approached. The acidity is usually expressed in terms of cubic centimeters of 0.1 *N* alkali required to neutralize the twenty-four-hour output of urine.

The reaction of urine is dependent upon the character of the diet. Accordingly, the titratable acidity may fall normally within a wide range of values (150–500 cc.). The average is probably about 300–350 cc. Even an alkaline urine (pH > 7) has a titratable acidity, for the color change of phenolphthalein is well over on the alkaline side (pH = 8.5). On diets rich in acid-forming foods (meat, fish, oatmeal, rice, wheat, egg yolk, prunes, etc.), a very acid urine is produced. Values of 600–900 cc. for titratable acidity may be obtained easily if sufficient quantities of such food are eaten. Most vegetables and fruits (oranges, potatoes, beans, raisins, apples, bananas, carrots, beets, etc.) are base-forming and yield an alkaline urine. This accounts for the fact that the urine of herbivorous animals is normally alkaline, whereas carnivorous animals ordinarily secrete an acid urine.

Urinary acidity bears a relationship to the excretion of ammonia, low acidity being associated with low values for ammonia, whereas urines having high acidities contain much larger quantities of ammonia. An exception to this is often observed in nephritis, where high acidity values are not always associated with correspondingly high values for ammonia.

Van Slyke and Palmer [14] have shown that men normally excrete the equivalent of about 6.0 cc. of 0.1 *N* organic acid per kilo of body weight in twenty-four hours. In starvation and in diabetes, the organic

[13] *Ibid.*, **65**, 197 (1925).
[14] *J. Biol. Chem.*, **41**, 567 (1920).

acid elimination is markedly increased. A similar alteration, commensurate with the changes in titratable acidity, is observed in the ammonia output in the urine. Increase in ammonia elimination may be produced experimentally by feeding acid (Fiske and Sokhey [15]). In a later experiment, Fiske [16] observed that when exceedingly large doses of acid were given there was relatively more loss of fixed base, and less of ammonia production, than when the dose was smaller; but for several days after the acid administration, the output of fixed base was lower than normal, whereas the ammonia elimination continued to be high, showing that there was a retention of fixed base at the expense of ammonia.

Blatherwick and Long [17] observed that drinking large amounts of orange juice resulted in the production of an alkaline urine, an increased organic acid excretion, and a decreased output of ammonia. The explanation of the increased organic acidity is that a certain amount of the citric acid escapes oxidation and is eliminated in the urine as citrate and thus increases the titration value for organic acids. However, this affects only a small part of the citric acid even when large amounts are taken; in fact, the authors state: "It was impossible to overreach the organism's ability to oxidize the contained citric acid even though the amounts (of orange juice) drunk in one day were the equivalent of 48 grams of acid." Accordingly, the excess of base in the orange juice in Blatherwick's experiments was sufficient to balance the organic acidity which escaped oxidation and to cause an alkaline urine, as well as a marked depression in the ammonia excretion.

The increased urinary acidity produced by eating prunes and cranberries is due to hippuric acid.

Hydrogen-ion Concentration. The *p*H may be taken as a truer index of urinary acidity than is afforded by titration. Determinations of *p*H of urine are usually made by means of indicators. The extreme range is usually given as *p*H 4.80–7.50, the average normal value being about 6.0. Deviations from this mean value are dependent on the character of the diet, a high-acid diet yielding urine of low *p*H, whereas on a low-acid diet the urine obtained has a high *p*H. Low *p*H values are found in pathological conditions, especially in diabetes and cardiorenal disorders.

Perhaps the most striking change in the reaction of the urine is that which occurs after meals, when the urine becomes less acid and may even acquire a neutral or alkaline reaction. This is referred to as the "alkaline tide," and has been attributed to the withdrawal of hydrogen ions, attending the formation of the hydrochloric acid of the gastric juice. Inasmuch as in conditions of anacidity (lack of HCl

[15] *Ibid.*, **63**, 309 (1925).
[16] *Ibid*, **67**, 385 (1926).
[17] *Ibid.*, **53**, 103 (1922); **57**, 815 (1923); see also C. Schuck, *J. Nutrition*, **7**, 679, 691 (1934).

formation) Hubbard, Munford, and Allen [18] did not observe an alkaline tide, it may be inferred that the secretion of gastric juice is an important factor in causing it. Possibly the moderate diuresis which occurs after meals is another factor. The increased acidity of the urine secreted during sleep has been referred to in another connection. A large number of persons show on awakening a marked urinary alkalinity which persists through the morning period (Hubbard, *et al.*). This "morning tide," is, however, unrelated to the secretion of acid by the stomach, nor is it connected with the taking of a meal.[19]

Composition of Urine. In view of all that has been written in the preceding chapters, it would be superfluous to explain why the composition of the urine is so variable. It will therefore be sufficient at this point to review the better-known constituents of normal urine and to indicate, though very roughly, the daily excretion of some of these.

TABLE L

ORGANIC CONSTITUENTS

Nitrogenous	Daily Output, grams	Non-nitrogeneous	Daily Output, grams
Urea	20.00	Glucose and other reducing carbohydrates	<1.0
Creatinine	1.70	Aromatic oxyacids	<0.1
Creatine (p. 425)		Oxalate	0.2
Ammonia	0.70	Acetone, acetoacetic acid	0.01
Uric acid	0.60		
Hippuric acid	0.50		
Indican	0.01		
Allantoin (p. 437)	(?)		

In addition to these are the pigments (urochrome, uropterine, urobilin, in traces normally), cystine and other neutral sulfur compounds (thiocyanate, taurine derivatives, ethereal sulfates, other than indican, such as skatoxylsulfuric acid, phenyl, and paracresolsulfuric acid, etc.) traces of oxaluric acid, organic phosphates, etc.

INORGANIC CONSTITUENTS

	Daily Output, grams		Daily Output, grams
Chloride, expressed as NaCl	10	Potassium	1.5
Phosphate, expressed as P_2O_5	2.5	Calcium	.2
Total sulfur, expressed as SO_3	1.6	Magnesium	.2
Sodium	5.0	Iron	.005

The urine also contains traces of silicate, fluoride, bicarbonate, copper, and zinc.

Composition of Urine in Relation to Diet. At this stage of the discussion, it may be profitable to consider how the composition of the

[18] *Am. J. Physiol.*, **68**, 207 (1924). *J. Biol. Chem.*, **101**, 781 (1933); *ibid.*, **84**, 191, 199 (1929); *Proc. Soc. Exptl. Biol. Med.*, **27**, 212, 327 (1929–30).

[19] For a review of the subject of "alkaline tide" the student is referred to C. E. Brunton, *Physiol. Rev.*, **13**, 372 (1933).

diet influences that of the urine. By this time, the student has no doubt become aware that the study of certain phases of metabolism requires a knowledge of the end-products appearing in the urine. The type of information which may be obtained from the quantitative analysis of urine will be illustrated by a few simple examples.

High- and Low-Protein Diet. Let us suppose that the student wishes to determine the effect of changing the amount of protein of the diet on the composition of the urine. He may proceed to do so by first examining the urine formed on a diet containing the amount of protein to which he is normally accustomed. The composition of the food should be known. This information may be obtained by analyzing the food, or it may be calculated from known data, since the composition of the common foodstuffs has been determined. On a given day the urine should be collected over a period of exactly twenty-four hours. This urine is then analyzed for the constituents to be given presently, as well as for any others which it may be desirable to determine.

Having performed this preliminary analysis, the student may now vary his protein intake. It is preferable that he remain on the experimental diet (high-protein intake, low-protein intake, etc.), not only on the day when the urine is collected, but for two or three days preceding this. The reason for beginning the experimental diet several days before collecting the urine is that an alteration in the diet does not always produce an immediate effect on the composition of the urine. There is often a lag in the elimination of the nitrogenous end-products of metabolism. This is referred to as the "nitrogen lag."

In Table LI are recorded the results of representative analyses of twenty-four-hour specimens of urine collected (subject, male, weighing 67 kg.).

(*a*) On a diet containing an ordinary amount of protein (equivalent to about 15 grams of nitrogen per day).

(*b*) On a diet containing more than the usual amount of protein (equivalent to about 25 grams of nitrogen, given in the form of meat and eggs, etc.).

(*c*) On a diet containing very little protein (cream, starch, butter, potatoes) but adequate as regards caloric requirements.

Urea nitrogen is usually 80–90 per cent of the total nitrogen, but when the total nitrogen is very low the urea nitrogen may be only 60–70 per cent of the total. On a high-protein diet, particularly on one containing meat, the output of total sulfur and phosphorus is increased, as well as the titratable acidity. The change in acidity influences the ammonia output, as indicated above. The increase in uric acid on the protein-rich diet and the decrease on the protein-poor diet are to be attributed to the presence of nucleic acid in the protein fed (part of it was meat). The undetermined nitrogen represents the nitrogenous constituents, other than those given, which are present in urine. The

most important of these are probably hippuric acid and purine bases. Like ammonia, hippuric acid is believed to be synthesized in the kidney. On a high-protein diet, there is usually a greater amount of intestinal putrefaction than otherwise occurs. This may account for the increase in ethereal sulfates on the protein-rich diet. No special significance need be attached to the changes in the elimination of chloride and water. When large quantities of food are consumed, an increased intake of salt and water is usually incidental.

TABLE LI

INFLUENCE OF PROTEIN INTAKE ON THE COMPOSITION OF URINE
(Daily output)

	Usual Protein Intake	Protein-rich Diet	Protein-poor Diet
Total nitrogen * (g.)	13 20	23.28	4.20
Urea nitrogen (g.)	11 36	20 45	2.90
Ammonia nitrogen (g.)	0.40	0.82	0.17
Creatinine nitrogen (g.)	0 61	0.64	0.60
Uric acid nitrogen (g.)	0 21	0.30	0 11
Undetermined nitrogen (g.)	0.62	1 07	0.52
Titratable acidity (cc. 0.1 *N*)	284 0	655 0	160.0
Total sulfur as SO_3 (g.)	2.65	3.55	0 86
Inorganic sulfate as SO_3 (g.)	2.16	2 82	0.64
Ethereal sulfate as SO_3 (g.)	0 18	0 36	0.11
Neutral sulfur as SO_3 (g.)	0 31	0 37	0.11
Total inorganic phosphate as P_2O_5 (g.)	2 59	4 07	1.06
Chloride as NaCl (g.)	12 10	15 10	9.80
Volume (cc.)	1260	1550	960

* Total nitrogen is determined by the Kjeldahl method For purposes of comparison, the concentrations of the nitrogenous constituents are expressed usually in terms of nitrogen. The analytical procedures are described in laboratory manuals of biochemistry.

High- and Low-Purine Diet. The influence of purine-free and purine-rich diets is indicated in the following table. In the experiments represented by the data contained therein, an attempt was made to maintain the total nitrogen intake at approximately the same level as on the day of the normal protein diet described above. The purine-rich diet consisted largely of glandular tissues (thymus, pancreas, and liver). On the low-purine diet, the most important changes were those involving the titratable acidity and the output of uric acid and phosphates. All three were increased on the purine-rich diet and diminished on the low-purine diet. These changes were due to the relative abundance, in the first case, of uric acid and phosphate precursors, and the relative lack of these in the second case. With the increased acidity on the high-purine diet, there was a corresponding rise in ammonia excretion. The high value for

undetermined nitrogen on this diet was due, no doubt, to an increased elimination of purine bases other than uric acid.

It will be observed that there was very little, if any, change in the creatinine values in this and in the preceding series of experiments, as might be expected from the fact that creatinine is derived from endogenous and not exogenous sources.

TABLE LII

INFLUENCE OF HIGH- AND LOW-PURINE DIETS ON THE COMPOSITION OF URINE
(Daily Output)

	High-Purine Diet	Low-Purine Diet
Total nitrogen (g.)	15.75	13.54
Urea nitrogen (g.)	12 97	11 88
Ammonia nitrogen (g.)	0 90	0 51
Creatinine nitrogen (g.)	0 61	0 60
Uric acid nitrogen (g.)	0.43	0 11
Undetermined nitrogen (g.)	0 84	0.44
Titratable acidity (cc. 0.1 *N*)	638	182
Total sulfur as SO_3 (g.)	3 64	2.00
Inorganic sulfate as SO_3 (g.)	2 81	1.53
Ethereal sulfate as SO_3 (g.)	0 46	0 22
Neutral sulfur as SO_3 (g.)	0 39	0 25
Total phosphate as P_2O_5 (g.)	3 94	1.40
Chloride as NaCl (g.)	13 20	12.80
Volume (cc.)	1620	1410

Fasting. Equally instructive are the changes that occur during starvation. The results in Table LIII are based upon analyses of urine collected during the first and fourth days of a fasting period.

It is obvious that, during the first day of the fasting period, sufficient glycogen was available to supply most of the energy requirements. Consequently, relatively little protein was broken down for this purpose, as shown by the data. Since glucose metabolism was taking place, there was complete conversion of creatine to creatinine. There was, likewise, complete oxidation of fatty acids, as evidenced by the fact that the urine did not contain abnormal quantities of acetone bodies. The excretion of ammonia, uric acid, sulfate, and phosphate, and the titratable acidity, were less than on the normal diet, owing to the diminished metabolism of amino acids and purines.

By the fourth day, the glycogen stores had been fairly well depleted, for there was on that day an increase in tissue breakdown (rise in total N), as well as incomplete conversion of creatine into creatinine and failure in fat combustion (appearance of large amounts of acetone bodies). The continued decrease in uric-acid elimination may be

TABLE LIII

INFLUENCE OF FASTING ON THE COMPOSITION OF URINE
(Daily Output)

	First Day of Fast	Fourth Day of Fast
Total nitrogen (g.)	7 08	14.40
Urea nitrogen (g.)	5 80	11.82
Ammonia nitrogen (g.)	0 21	1.32
Creatinine nitrogen (g.)	0 59	0.44
Creatine nitrogen (g.)	.. ..	0.16
Uric acid nitrogen (g.)	0 15	0.08
Undetermined nitrogen (g.)	0 33	0.58
Titratable acidity (cc. 0.1 *N*)	176	720
Total sulfur as SO_3 (g.)	1.22	2.01
Total phosphate as P_2O_5 (g.)	1.71	1.14
Chloride as NaCl (g.)	5 20	1.26
Acetone bodies (g.)	(trace)	3.86
Volume (cc.)	860	880

explained partly on the basis of diminished nuclear metabolism and uric-acid retention in the blood, as has been observed by Lennox.[20] Commensurate with this change, there was a decrease in the excretion of phosphates. Nevertheless, the titratable acidity was higher than normal, owing to the acetone bodies. Accordingly, there occurred a corresponding increase in the formation and excretion of ammonia. Creatine appeared in the urine at the expense of creatinine. Chloride elimination decreased to a low level.

Pathological Constituents of Urine. *Glucose.* Mellituria usually refers to the occurrence of reducing carbohydrate in urine; *glycosuria* more specifically to the presence of glucose. Even the normal individual excretes glucose and perhaps other reducing sugars, but the amount is small, the total usually not exceeding 1 gram per day. There are, however, persons with a tendency to a more pronounced glycosuria, usually in the early afternoon, a condition, apparently benign, which Harding [21] has described as *afternoon glycosuria.* Exercise may cause glycosuria, especially if it involves emotional stress, as on the football field.

There is also the condition called *renal glycosuria,* in which the excretion of glucose may be considerable, even though the blood concentration remains normal. Renal glycosuria is ascribed to a low renal

[20] *J. Biol. Chem.*, **66**, 521 (1925).

[21] For a discussion of the sugars of urine compare V. J. Harding, and D. L. Selby, *Biochem. J.*, **25**, 1815 (1931) and E. S. West, *et al.*, *ibid.*, **26**, 1720, 1728, 1742 (1932). H. T. Edwards, T. K. Richards, and D. B. Dill, *Am. J. Physiol.*, **98**, 352 (1931); F. A. Hellebrandt, *ibid.*, **101**, 357 (1932).

threshold for glucose and seems to be a benign disorder. Its familial or hereditary nature has been commented on by a number of writers.[22]

Glycosuria may occur after eating uncommonly large amounts of sweets. This form, described as *alimentary glycosuria,* is especially conspicuous in persons with a subnormal renal threshold. Although alimentary glycosuria is said to occur in presumably healthy individuals, its persistence in a given subject should call for careful study in order to rule out diabetes.

Of the utmost importance clinically is the form of glycosuria associated with diabetes. This is accompanied by an elevation in blood sugar.

Fructose. Fructose occurs often, together with glucose, in diabetic urine, but has also been observed in the urine of non-diabetic individuals. Essential fructosuria is a comparatively rare anomaly [23] and is judged by Silver and Reiner [24] to be probably an inborn error of metabolism in which there is deficient utilization of fructose by the liver. The metabolism of other carbohydrates is apparently undisturbed.

Galactose. The occurrence of galactose in the urine in infancy has been described by Mason and Turner.[25] The condition has been attributed to incomplete conversion of galactose into glycogen by the liver. Failure to remove milk from the mammary glands of lactating animals may lead to the appearance of lactose in the urine. Lactosuria is occasionally observed during pregnancy and lactation and is somewhat more frequent during the weaning period.[26]

The occurrence of pentosuria has been mentioned elsewhere (p. 49).

Albuminuria or Proteinuria. Albuminuria is the older terminology, but the term proteinuria is more accurate, as urine may contain globulin and perhaps other proteins, as well as albumin. Normally, urine contains about 20 to 50 mg. of protein per liter, an amount not detectable by the usual tests. A more conspicuous proteinuria occurs in a small proportion of individuals, predominantly in adolescents and youths, in whom there may be no actual evidence of kidney disease. Certain individuals in assuming an upright position, more especially when standing in the military position of attention, develop albuminuria. The terms *orthostatic* and *postural* albuminuria are used to describe this condition. There seems to be some association between postural albuminuria and lordosis, and possibly also a connection between lordosis and pressure phenomena on the kidney causing renal venous stasis. Proteinuria also occurs commonly in febrile diseases.

However, in none of these conditions is the proteinuria very severe; as a rule the amount of protein present is barely perceptible, and even

[22] F. P. Weber, *Lancet,* **2**, 71 (1931); M. S. Brown and R. Poleshuck, *J. Lab. Clin. Med.,* **20**, 605 (1934–35).

[23] A. Marble and R. M. Smith, *J. Am. Med. Assoc.,* **106**, 24 (1936).

[24] *Arch. Internal Med.,* **54**, 412 (1935).

[25] *Am. J. Diseases Children,* **50**, 359 (1935).

[26] O. Watkins, *J. Biol. Chem.,* **80**, 33 (1928).

in orthostatic albuminuria the daily excretion is often only 0.1 gram per day, though it may amount to as much as 3 grams. Much larger quantities of protein are excreted in hemorrhagic Bright's disease and especially in degenerative Bright's disease, or nephrosis, in which condition a daily loss of protein of 10 grams is not uncommon, and it may amount to 20 and even 30 grams. There is substantial evidence that the urinary proteins have their origin in the blood and correspond to the protein fractions of the blood serum (p. 233).[27]

Bence-Jones Proteinuria. Bence-Jones protein, named after its discoverer, was originally detected in the urine of a patient with multiple myelomata. The protein is characterized by its insolubility at moderate temperatures (40–60° C.). As the temperature of the solution (or urine) is raised, the protein redissolves; on cooling, the protein reappears. According to recent work by Meyler,[28] Bence-Jones protein is a normal constituent of bone marrow and of white blood corpuscles. Destruction of either increases the amount of the protein in circulation. Apparently a large part is either utilized or destroyed. It is only when excessive amounts of Bence-Jones protein are liberated that a part is excreted in the urine. However, cases are on record in which the daily output exceeded 50 grams. When the quantity excreted is large and the condition is of long duration, anatomical injury to the kidneys may be anticipated.[29] Apart from its excretion in patients with multiple myelomata, Bence-Jones protein has been observed in Hodgkin's disease, leukemia, lymphosarcoma, and empyema.

Bile Pigments. The urine normally contains very little if any bilirubin, but in jaundice considerable amounts of this pigment may be excreted, along with bile acids. In the intestine, bilirubin is normally converted into urobilinogen, which is reabsorbed and may occur in the urine in traces. On exposure to air, urobilinogen is oxidized to urobilin. In certain diseases of the liver, in hemolytic jaundice and other conditions associated with increased bile pigment metabolism, the urine may contain comparatively large amounts of bilirubin, urobilin, and urobilinogen. On the other hand, in complete obstructive jaundice, owing to the fact that the bile does not gain access to the intestine, urobilinogen and urobilin are not formed and therefore do not occur in the urine.

[27] E. M. Widdowson, *Biochem. J.*, **27**, 1321 (1933); see also A. Hiller, J. F. McIntosh, and D. D. Van Slyke, *J. Clin. Investigation*, **4**, 235 (1927).

A. S. Alving and A. E. Mirsky, *J. Clin. Investigation*, **15**, 215 (1936), have investigated the serum and urine protein fractions in a case of nephrosis. The serum albumin was separated into two fractions; one had the same cystine content as normal serum albumin, the other contained less cystine than either normal serum albumin or serum globulin. The urinary albumin was found to contain more cystine than the corresponding fraction (total albumin) of the serum, but less than normal serum albumin. Evidently the kidneys in nephrosis are capable of exerting a selective action in filtering out more of the high-cystine albumin than of the albumin of low cystine content.

[28] *Arch. Internal Med.*, **57**, 708 (1936).

[29] W. D. Forbus, W. A. Perlzweig, *et al.*, *Bull. Johns Hopkins Hosp.*, **57**, 47 (1935).

Melanin, etc. The urine of persons with melanotic tumors, on exposure to air, gradually turns dark brown or black, owing to the presence of an oxidizable substance which on oxidation yields a black pigment (melanin). The same change occurs, but more rapidly on adding a few drops of ferric chloride solution to the urine. Another pigment found in certain pathological conditions (pulmonary tuberculosis, typhoid fever, nephritis, etc.) is urorosein, present as a chromogen which has been shown to be indoleacetic acid. Its occurrence is demonstrated by the development of a rose-red color when urine (10 cc.) is treated with concentrated HCl (5 cc.) and a few drops of KNO_2. The pigment thus formed may be extracted with isoamyl alcohol. Homogentisic acid is present in the urine of alcaptonurics, and cystine in that of cystinurics.

Creatine and the acetone bodies have been discussed elsewhere. The occurrence of porphyrinuria has also been mentioned (p. 247).

Sediment. The particulate matter of urine may be collected by centrifuging. Among the constituents that settle out are calcium phosphate, uric acid, and urates. Calcium carbonate occurs in the urine of herbivorous animals, but very rarely in human urine. Calcium oxalate crystals are frequently observed, especially after apples or sweet potatoes have been eaten. Crystals of leucine, tyrosine, and cystine are said to be present occasionally, even in normal urine, but usually the occurrence of the first two is associated with destructive changes in the liver (p. 401) and of the last with cystinuria (p. 390).

Among the cellular elements are epithelial cells and cell débris derived from the lining epithelium of the urinary tract. An occasional pus cell (polymorphonuclear leukocyte) may be found. These constituents are markedly increased in inflammatory conditions of the pelvis, kidney, ureters, bladder, or urethra. Spermatozoa may also be present in urine.

Microscopic examination of urinary sediment is often made with the object of determining the presence or absence of casts. These are derived from the renal-tubular epithelium and are usually cylindrical in shape, having parallel sides and rounded ends. Casts are classified according to their morphological characteristics. There are the so-called hyaline, granular, epithelial waxy and fatty casts. These are described in detail in textbooks devoted to clinical pathology. The presence of casts in the urine, together with a positive test for albumin, is diagnostic of renal disorder. The occurrence of red blood cells may indicate a lesion anywhere in the urinary tract, and is of particular value in the diagnosis of acute hemorrhagic nephritis.[30]

Renal Function. Of the numerous clinical procedures that have been introduced for the estimation of the functional capacity of the kidneys, perhaps the most widely used is the phenolsulfonphthalein

[30] Normal urine is not entirely devoid of red blood cells and casts, as has been shown by Addis. Consult, T. Addis and J. Oliver, "The Renal Lesion in Bright's Disease," New York, 1931. See also H. Gibbons, *Arch. Internal Med.*, **54**, 758 (1934).

test. When a small amount of this dye (6 mg.) is injected, from 60 to 85 per cent is normally recovered in the urine within the succeeding two hours. A markedly diminished elimination especially during the first 30 minutes is an indication of severe renal insufficiency. This test, though of considerable clinical value, has many limitations.[31]

Concentration and Dilution Tests. The diseased kidney loses its ability to form either a very concentrated or a very dilute urine, in consequence of which the variation in specific gravity is usually small. This relative fixation in specific gravity has been utilized clinically in the estimation of renal efficiency. A description of the different tests is outside the scope of this book, but the principles involved in a few of them may be briefly stated.

The various concentration tests consist essentially in determining the effect of water deprivation for a given period (usually about 16 hours) on the specific gravity of the urine formed toward the conclusion of that period. Under the conditions prescribed, the normal individual, excepting the very aged, is capable of producing a urine of specific gravity 1.026, or higher. In advanced kidney disease the specific gravity may fall far short of this value.

In the dilution tests, the subject is given a definite amount of water to drink (1200 cc., for example). Urine is collected at frequent intervals thereafter, and the volume and specific gravity of each specimen are measured. The normal person usually eliminates all of the 1200 cc. within two or three hours. The urine is dilute, and the specific gravity of the largest specimen approaches 1.002. Under the same conditions, the individual with renal impairment may form only a small volume of urine, perhaps not more than 200 or 300 cc. The specific gravity will therefore be comparatively high ($1.010\pm$).[32]

Urea Clearance. Studies of the rate of urea excretion by numerous workers have established a certain correlation between the concentrations of urea in the blood and urine. On the basis of such relationships as have been obtained, various formulas have been proposed for the calculation of renal efficiency from observed data.

The procedure developed in Van Slyke's laboratory [33] depends essentially on this principle. To obtain the necessary data, two one-hour specimens of urine and one specimen of blood are required. The best time for conducting the test is between the hours of breakfast and

[31] For a description of various renal function tests the reader is referred to A. M. Fishberg, "Hypertension and Nephritis," Lea & Febiger, Philadelphia, 3d edition, 1934, Chapter 2. Consult also E. M. Chapman and J. A. Halsted, *Am. J. Med. Sci.*, **186**, 223 (1933).

[32] For further details consult A. S. Alving and D. D. Van Slyke, "The Significance of Concentration and Dilution Tests in Bright's Disease," *J. Clin. Investigation*, **13**, 969 (1934); R. H. Freyberg, "The Choice and Interpretation of Tests of Renal Efficiency," *J. Am. Med. Assoc.*, **105**, 1575 (1935).

[33] E. Möller, J. F. McIntosh, and D. D. Van Slyke, *J. Clin. Investigation*, **6**, 427, 485 (1928); E. M. MacKay, *ibid.*, 505 (1928).

lunch (10 to 12 A.M.), when the excretion is least liable to fluctuation. The bladder may be emptied at 10 o'clock and a glass of water taken. The subject then remains quiet and urine is collected in two succeeding periods of one hour each. If the interval is somewhat shorter or longer, no error is introduced, provided the calculations for the excretion per minute are based on the actual time. The blood is taken within a few minutes after the first urine specimen is collected.

The urea content is determined in each specimen of urine and the values averaged. The blood is likewise analyzed for this constituent.

If the urine volume exceeds 2 cc. per minute, the " maximum clearance," C_m, is calculated.

$$C_m = \frac{UV}{B}$$

where U = concentration of urea in the urine (per unit volume, such as 100 cc.), B = concentration of urea in the blood, and V = the urine volume in cubic centimeters per minute.[34]

If the urine volume (corrected volume for children) is less than 2 cc. per minute, the " standard clearance," C_s, is determined.

$$C_s = \frac{U\sqrt{V}}{B}$$

where $\sqrt{V}$ is the square root of the volume.

The maximum clearance varies within relatively wide limits (64–99 cc.) even in normal individuals, the accepted average being 75 cc. This means that in passing through the kidneys an average of 75 cc. of blood are cleared of urea per minute.

The standard clearance likewise varies within a wide range of values (41–65 cc.), the accepted average being 54 cc.

[34] In children a correction for body size is required. The volume is multiplied by the factor 1.73/body area in square meters. This correction is also applied to adults of unusually short stature. Consult J. F. McIntosh, E. Möller, and D. D. Van Slyke, *J. Clin. Investigation*, 6, 467 (1928). See also J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," Vol. 1, p. 345, and Vol. II, p. 564.

In most cases it makes little difference whether the ammonia output is taken into account, but in certain conditions, such as those associated with acidosis (diabetes, starvation, etc.), the ammonia output may be of significance in the calculations. It has therefore been proposed by Van Slyke and associates, *J. Clin. Investigation*, 14, 901 (1935), that the calculations should be based, not on the values for urea N, but on those for urea plus ammonia N. The necessity of separate determinations of urea and ammonia is thus obviated.

It has been shown by Chesley, *J. Clin. Investigation*, 16, 653 (1937), that, when the urine flow falls below a certain critical volume, which for adults is about 0.35 cc. per minute, the square-root formula for calculating the standard urea clearance gives erroneously low results. Accordingly, he has recommended that when the urine collected in an hour's clearance test falls below 20 cc., the test should be discarded.

However, in kidney disease, departures from the normal are usually very conspicuous.

The results may be expressed in terms of the normal renal function. by applying either of the following formulas, depending on whether the maximum or standard urea clearance has been determined.

$$\text{Percentage of normal function} = \frac{C_m \times 100}{75}$$

$$\text{Percentage of normal function} = \frac{C_s \times 100}{54}$$

Creatinine Excretion. Creatinine has been considered as a non-threshold substance, or relatively so. On the assumption that this constituent is neither absorbed nor secreted by the tubules, its content in the urine may be taken as a measure of glomerular filtration. On this basis, Rehberg [35] introduced a test which depends on the rate of excretion of creatinine after a definite amount is ingested. By comparing the creatinine concentration of the plasma and urine at different intervals, a measure is obtained, not only of the kidney's capacity to excrete this constituent, but presumably also of the rate of glomerular filtration.[36] In the majority of his experiments, Rehberg found the excretion per minute to be equal to the amount of creatinine contained in 110–150 cc. of plasma. Hence this value may be considered to represent the volume of glomerular filtrate. Inasmuch as the volume of urine per minute is relatively small, usually 1–2 cc., the difference may be taken to represent the volume of fluid reabsorbed by the tubules.

In a study by Hayman,[37] the "creatinine clearance" in 59 normal subjects (130 observations) was found to average 148 cc. per minute. As compared with this were the conspicuously low values (as low as 0.6 cc.) obtained in cases of renal insufficiency.

[35] *Biochem. J.*, **20**, 447 (1926).

[36] See, however, A. W. Winkler and J. Parra, *J. Clin. Investigation*, **16**, 859, 869 (1937).

[37] J. M. Hayman, J. A. Halsted, and L. E. Seyler, *J. Clin. Investigation*, **12**, 861 (1933); see also R. Dominguez and E. Pomerene, *J. Biol. Chem.*, **104**, 449 (1934).

CHAPTER XVI

INTERNAL SECRETIONS OR HORMONES

The glands of internal secretion, or *endocrine organs*, will be considered in this chapter mainly from the standpoint of the chemistry of their physiologically active substances and of their effect in regulating the various activities of the animal organism. Certain glandular secretions, not those with which we are concerned here, are transported by means of ducts. The gastric and pancreatic juices and the saliva are familiar examples of such secretions. In contrast to these it has been discovered that certain glands pour their products directly into the blood stream. These *ductless* glands and their secretions are the subject of the present chapter. The internal secretions are of the utmost importance to the animal body because of their action in controlling and integrating its manifold activities; in fact, certain secretions seem to be indispensable to life.

Although the subject of endocrinology may be said to have an earlier history, nevertheless, the work done by Bayliss and Starling [1] on *secretin*, in 1902, is usually regarded as marking the beginning of the modern development of this important branch of physiology and biochemistry. Bayliss and Starling recognized that substances of the type of secretin were probably chemical in nature, and, since such substances appeared to stimulate or arouse organs and tissues to activity, they suggested the term *hormone*, from the Greek, meaning "I rouse to activity."

Since a hormone is conventionally defined as a substance formed in one organ and carried to another organ where it sets up definite physiological activity, even such compounds as urea and carbon dioxide might perhaps be regarded as hormones. Urea has its origin in the liver and stimulates the kidney; carbon dioxide exerts an effect on the respiratory center. It is difficult at present to make an absolute rule as to what to include and what to exclude under the definition; nevertheless, the substances mentioned are not classed with the hormones. The internal secretions with which we are principally concerned are those of the pancreas, thyroid, parathyroids, adrenals, sex glands, placenta, and hypophysis. Of these, the pancreas and the generative glands have both internal (ductless) and external (duct) secretions. Whether the liver should also be included, in view of the occurrence in this organ of a

[1] *J. Physiol.*, **28**, 325 (1902).

substance which is concerned with blood formation and which is a specific curative agent for pernicious anemia, is at present a question on which there is lack of agreement. As yet, it has not been established that the active constituent is in the nature of a hormone. The anti-anemic principle which is stored principally in the liver, but also in the kidney, appears to be a combination of the so-called *intrinsic* factor found in the gastric mucosa (p. 182) and of an *extrinsic* factor contained in certain foods, such as beef muscle and yeast.[2] The intrinsic factor is thermolabile, being inactivated at about 70° C., and has other properties which suggest that it may be an enzyme. Finally, we should consider the internal secretions of the gastric and intestinal mucosa which are concerned with the function of digestion.

Hormones of the Gastrointestinal Tract.[3] These have been discussed in the chapter on digestion and will therefore be reviewed only very briefly at this point.

As has been stated elsewhere (p. 171), extracts of the pyloric mucous membrane, when injected into the circulation, cause an increased secretion of gastric juice, an effect that has long been attributed to a specific secretagogue, in the nature of a hormone, called *gastric secretin* or *gastrin*. According to the work of Ivy and associates this hormone is identical with histamine.[4]

Secretin is of special interest because its discovery by Starling in extracts of the duodenal mucosa in no small measure stimulated research in this important field of physiological chemistry. Its relation to pancreatic secretion is well established. In all probability, secretin is a relatively simple substance. Its isolation in crystalline form has been reported.[5] Secretin is diffusible through parchment and soluble in acids and in 95 per cent alcohol (which indicates that it is not a protein); it is not destroyed by heat, but is sensitive to the action of alkali, in which it is also soluble. The chemical nature of secretin remains to be determined.[6]

Associated with secretin in the intestinal mucosa is another hormone, *cholecystokinin*, which is apparently concerned with the contractility and evacuation of the gall bladder. Extracts of the intestinal mucosa have been prepared which seem to be free from secretin and which when injected intravenously into dogs exert this specific effect on the gall bladder. According to Ivy,[7] its discoverer, *cholecystokinin* is closely related to, but probably not identical with, secretin. It is

[2] Consult W. B. Castle, "The Etiology of Pernicious and Related Macrocytic Anemias," *Harvey Lectures*, **30**, 37 (1934–35).

[3] The subject has been recently summarized by A. C. Ivy, *J. Am. Med. Assoc.*

[4] J. Sacks, A. C. Ivy, J. P. Burgess, and J. E. Vandolah, *Am. J. Physiol.*, **101**, 331 (1932).

[5] E. Hammarsten, E. Jorpes, and G. Ågren, *Biochem. Z.*, **264**, 272 (1933).

[6] E. U. Still, "Secretin," *Physiol. Rev.*, **11**, 328 (1931).

[7] A. C. Ivy, "Factors Concerned in the Evacuation of the Gall Bladder," *Medicine*, **11**, 345 (1932).

formed in the upper intestinal mucosa when in contact with acid, and probably fatty acids and other substances that cause secretin formation.

Enterogastrone. The presence of undigested fat in the intestine is known to inhibit gastric motility and secretion.[8] The fact that such inhibition was obtained in a transplanted gastric pouch suggested a humoral mechanism, a conclusion supported by the work of several laboratories. It is believed that the inhibitory effect is due to a specific substance in the intestinal mucosa that is activated in the presence of fat (p. 173).

Insulin; Hormone of the Pancreas. One of the most fascinating and inspiring chapters in the history of medicine began in the year 1889 when von Mering and Minkowski [9] in some experiments involving the removal of the pancreas from dogs discovered that this produced a condition similar to diabetes observed in man. It had previously been suspected that the pancreas might be related in some way to this condition as lesions in this organ were occasionally found in severe cases. As in human diabetes, the most prominent symptoms observed in the experimental form of pancreatic diabetes were the appearance of sugar in the urine, thirst, hunger, emaciation, and death in coma.

Some years later, Minkowski[10] grafted a piece of pancreas under the skin of a dog and subsequently removed the pancreas of the animal, leaving the grafted piece which by this time had established circulation with the blood. It was found that in this way diabetes could be prevented or delayed for several months. Similar experiments were performed by Hédon.[11]

Among the numerous experiments which ultimately led to the adoption of the idea that the action of the pancreas in regulating carbohydrate metabolism was due to a hormone, may be mentioned that of Forschbach,[12] who made an anastomosis of the blood supply of two dogs and then removed the pancreas of one of the animals. Neither dog developed diabetes. Interpreted in the light of our present knowledge, the pancreas of the unoperated dog obviously supplied sufficient hormone to take care of the metabolism of both animals.[13]

[8] T. Kosaka and R. K. S. Lim, *Chinese J. Physiol.*, **7**, 5 (1933).

[9] *Arch. exptl. Path. Pharm.*, **26**, 371 (1890).

[10] *Ibid.*, Supplementary volume, 1908, p. 399.

[11] *Arch. physiol.*, 5e série, **6**, 269 (1894).

[12] *Arch. exptl. Path. Pharm.*, **60**, 131 (1909).

[13] Another experiment is that of Carlson, *J. Biol. Chem.*, **17**, 19 (1914), who depancreatized a number of pregnant bitches and observed no glycosuria in these animals, presumably because of the functional activity of the pancreases of the fetuses. Lusk, however, in "The Science of Nutrition," 1917 edition, p. 453, cites some experiments of Murlin, who found that such dogs have diabetic respiratory quotients (0.69). Murlin suggests that the absence of glucose from the urine may have been due to the retention of carbohydrate by the fetuses. According to more recent observations of Schlossmann, in *Arch. exptl. Path. Pharm.*, **159**, 213 (1931), the placenta is impermeable to insulin.

The observations of Knowlton and Starling[14] were likewise suggestive of a hormone mechanism. These workers demonstrated an increase in the consumption of glucose by a heart taken from a depancreatized animal and perfused with blood from the same animal, when there was added to the blood an extract prepared from the pancreas.

The Islands of Langerhans. Pancreatic tissue contains certain structures consisting of clumps of cells which differ in appearance and staining reactions from the remaining acinous or secreting epithelium. Because of their *insular* appearance, these structures have been named the *islands of Langerhans.*[15] Even before there was any certainty that the pancreatic hormone was formed in these islands, the belief grew up that this was the case. In fact, the hormone was named *insulin* by Sir Edward A. Schäfer in 1916 when its existence was still hypothetical. However, there was available some evidence to show that the lesions of the pancreas in severe diabetes were limited to the islets of Langerhans, but the value of these observations was more or less neutralized by contradictory observations. The degeneration of the acinous tissue of the pancreas, with relatively little injury to the insular cells, may be brought about by ligating the ducts from the pancreas. This was accomplished by several workers as early as 1900 and is of historical interest in connection with the important experiments of Banting to be described shortly.

Thirty years ago the outstanding problem of diabetes was clearly defined. It was a question of isolating and of determining the chemical nature of the pancreatic hormone. The first part of the problem was solved in 1922 by a group of active students in Macleod's laboratory at the University of Toronto. Guided by the work of earlier investigators whose pancreas extracts were usually toxic or inactive, owing partly to the presence of trypsin, Banting and Best[16] proceeded to prepare active preparations in which the effect of trypsin would be eliminated. By ligating the pancreatic ducts in dogs they succeeded in producing considerably more degeneration of the acinous cells than of the insular tissue. After a few weeks the dogs were depancreatized: Extracts prepared from these pancreases, when injected subcutaneously or intravenously into normal and diabetic animals, proved to be very effective in causing a reduction of the blood sugar and in otherwise relieving the symptoms of diabetes. At about this time Collip joined Banting and Best in their work and in a very short time developed methods for the preparation of extracts, first from fetal calf pancreases

[14] *J. Physiol.*, **45**, 146 (1912).

[15] The first to describe these structures was Langerhans (Inaugural Diss., Berlin, 1869). More recent studies have disclosed that there are three types of cells in the islands of Langerhans, the so-called alpha, beta, and gamma cells. The granular beta cells which occupy the periphery of the islets are believed to be the source of insulin.

[16] *Am. J. Physiol.*, **59**, 479 (1922); *J. Lab. Clin. Med.*, **7**, 251, 464 (1922). (Numerous other papers.)

and subsequently from ordinary beef glands, that were suitable for use in human diabetes. Thus was inaugurated a new era in the treatment of diabetes.

It would be beyond the scope of this book to review the enormous amount of work that has been done on insulin since its discovery by Banting and his associates; nor is it possible to enter here into a discussion of the clinical aspects of the problem. A review of the earlier phases of the subject has been prepared by J. J. R. Macleod,[17] and further information may be found in the current medical and biochemical literature. Nevertheless, some of the more important features of the problem will be considered briefly.

Source and Preparation. Insulin is widely distributed in both vertebrates and invertebrates. The main source at present is the pancreas of cattle. Fetal pancreases, lacking in functional acinous tissue, yield active preparations of insulin.[18] Collip's [19] method for preparing insulin consisted in fractional precipitation with alcohol. Various modifications have been proposed, among which may be mentioned that of Doisy, Somogyi, and Shaffer,[20] which consists in the further purification of insulin by precipitation at the isoelectric point (*p*H 5.0–6.0). Another method is that devised by Dudley.[21] It consists in precipitating the insulin from solution with picric acid as the picrate. It is subsequently converted to the hydrochloride and washed free from fat and certain other impurities with acetone and ether, in which insulin hydrochloride is insoluble. Dodds and Dickens [22] have modified Dudley's procedure. According to their method the finely minced pancreas is mixed with picric acid. This combines with the insulin to form the picrate, which is extracted with acetone. The extract is evaporated and the residue again extracted with ether to remove the fat and picric acid. The picrate is then converted to the hydrochloride and further purified as in Dudley's method.[23]

The occurrence of insulin in animal tissues other than the pancreas, and of insulin-like substances in plants, has been reported by many

[17] *Physiol. Rev.*, **4**, 21 (1924); "Carbohydrate Metabolism and Insulin," Longmans, Green & Co., 1926.

[18] A. M. Fisher and D. A. Scott, *J. Biol. Chem.*, **106**, 305 (1934), found that the insulin content of cattle pancreas diminishes with age. In 6- to 8-month old calves, the pancreas contained 5–6 times the amount present in 7- to 9-year-old cows, but only half the amount contained in 5- to 7-month-old fetal pancreas and only about a third of the amount found in the pancreas of cattle fetuses under 5 months.

[19] *Trans. Roy. Soc. Canada*, **16**, 18 (1922).

[20] *J. Biol. Chem.*, **55**, xxxi (1923).

[21] *Biochem. J.*, **17**, 376 (1923).

[22] *Brit. J. Exptl. Path.*, **5**, 115 (1924).

[23] A more detailed summary of the methods of preparing insulin is given by B. Harrow and C. P. Sherwin, "The Chemistry of Hormones," Williams & Wilkins, Baltimore, 1934. See also H. Jensen and E. A. Evans, *Physiol. Rev.*, **14**, 188 (1934).

workers, but this has been recently questioned by Best and associates.[24]

Chemical Properties of Insulin. The first work on insulin indicated that it might be a protein-like substance. Banting and Best [16] determined that insulin is readily destroyed by trypsin, an observation soon confirmed by Dudley,[21] who showed, moreover, that pepsin produces the same effect. This behavior explains, no doubt, the ineffectiveness of insulin when given by mouth.

Important advances in our knowledge of the chemistry of insulin have emanated from the laboratory of J. J. Abel [25] at the Johns Hopkins University, where methods for preparing highly purified, optically active, crystalline insulin were first developed. Crystallization was obtained at a *p*H of 5.55 to 5.65, which is approximately the isoelectric point of insulin. Repeated recrystallization of crude preparations of beef insulin and later of fish insulin, using widely different methods, yielded a uniform product, both as to crystalline structure, chemical composition, and physiological potency.[26] The identity of insulin from various sources (beef, hog, sheep, and fish pancreases) was further demonstrated by the work of Scott.[27] All these preparations were of similar crystalline structure, possessed the same physiological activity (approxi-

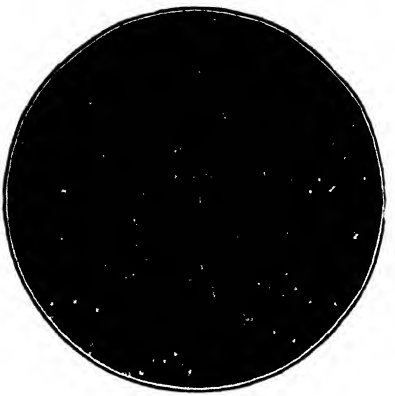


FIG. 34.—Crystalline Insulin. (Reproduced from a photomicrograph kindly furnished by Professor John J. Abel.)

[24] C. H. Best, C. M. Jephcott, and D. A. Scott, *Am. J. Physiol.*, **100**, 285 (1932). This paper contains a comprehensive bibliography of the subject in question.

[25] J. J. Abel, *Proc. Natl. Acad. Sci. U. S.*, **12**, 132 (1926); Abel, E. M. K. Geiling, C. A. Rouiller, F. K. Bell, and O. Wintersteiner, *J. Pharmacol*, **31**, 65 (1927); V. du Vigneaud, H. Jensen, and Wintersteiner, *ibid.*, **32**, 367, 387 (1927–28); du Vigneaud, Geiling, and C. A. Eddy, *ibid.*, **33**, 497 (1928); H. Jensen, Wintersteiner, and Geiling, *ibid.*, **36**, 115 (1929). H. Jensen and E. A. Evans, *Physiol. Rev.*, **14**, 188 (1934).

[26] See also C. R. Harington and D. A. Scott, *Biochem. J.*, **23**, 384 (1929). These investigators found certain commercial preparations of amorphous insulin with a potency almost as high as that of crystalline insulin. This would indicate that the preparation of the crystalline product is not a matter of isolating a highly active substance from a crude mixture, but rather a process analogous to the crystallization of a protein.

Scott has prepared crystalline insulin according to the methods of Abel and of Harington and Scott and found it to contain zinc. He pointed out that if steps are taken to remove the metals from crystalline insulin, the resulting material cannot be crystallized unless one of the following metals is added: cadmium, zinc, nickel, cobalt. *Biochem. J.*, **28**, 1592 (1934).

[27] *J. Biol. Chem.*, **92**, 281 (1931).

mately 24 international units per milligram; see p. 470), and contained the same amount of sulfur (about 3.2 per cent) that was found by Abel and associates on analyzing their preparations. Tenfold recrystallization of insulin did not alter its potency or other properties.

The isoelectric point of *crystalline* insulin is at *p*H 5.3–5.35, as determined by Wintersteiner and Abramson,[28] employing electrophoretic methods.

Analysis of crystalline insulin (Jensen and Wintersteiner)[29] has revealed the following distribution of amino acids (in per cent): tyrosine 12, cystine 12, glutamic acid 21, leucine 30, arginine 3, lysine 2. Crystalline insulin appears to contain no tryptophane.[30]

The weight of evidence at present favors the view that insulin is a protein and that the crystalline product described represents the hormone in its purest and most active form. The molecular weight, according to Sjögren and Svedberg,[31] is 35,100.[32]

Insulin heated to 100° C. with dilute hydrochloric acid (0.1 *N*) forms a coagulum which is physiologically inert. However, the inactivation is reversible, for neutralization with alkali yields a product which has approximately the same activity as the original insulin, although it cannot be crystallized. When insulin is similarly treated with alkali, both sulfur and ammonia are split off and it is irreversibly inactivated. Irreversible inactivation of insulin apparently results from the loss of sulfur.

Aldehydes, acetic anhydride, and reducing agents inactivate insulin. Indeed even so mild a reducing agent as cysteine exerts this effect,[33] presumably through the reduction of the disulfide (—S—S—) bond, upon which according to one school of investigators the physiological activity of the hormone depends.[34]

Jensen[35] believes that the characteristic constituent of insulin is a compound of glutamine and cystine having either of the following chemical structures:

[28] *J. Biol. Chem.*, **99**, 741 (1932–33); see also F. O. Howitt and E. B. R. Prideaux, *Proc. Roy. Soc.* (*London*), *B*, **112**, 13 (1932).

[29] *J. Biol. Chem.*, **98**, 281 (1932).

[30] T. D. Gerlough and R. W. Bates, *J. Pharmacol.*, **45**, 19 (1932).

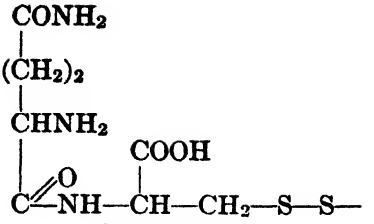
[31] *J. Am. Chem. Soc.*, **53**, 2657 (1931).

[32] According to analysis and on the basis that the insulin molecule contained one atom of sulfur, the formula $C_{45}H_{69}O_{14}N_{11}S$ was assigned by Abel. But, inasmuch as it was shown by du Vigneaud (*J. Biol. Chem.*, **75**, 393 [1927]) that insulin contains a disulfide linkage, the empirical formula was revised to $C_{90}H_{138}O_{28}N_{22}S_2$ (mol. wt. about 2040), it being understood that the actual molecule was probably larger, being a multiple of this value.

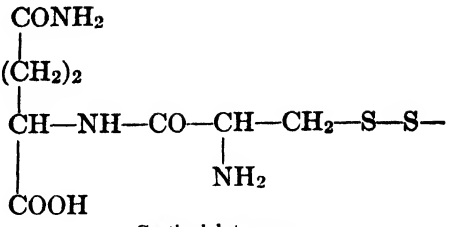
[33] V. du Vigneaud, A. Fitch, E. Pekarek, and W. W. Lockwood, *J. Biol. Chem.*, **94**, 233 (1931–32); O. Wintersteiner, *ibid.*, **102**, 473 (1933).

[34] Insulin does not lose its activity completely when a portion of the S—S groups is reduced. K G. Stern and A. White believe that only one or two dithio linkages in insulin have a special function. *J. Biol. Chem.*, **117**, 95 (1937).

[35] *Physiol. Rev.*, **14**, 188 (1934).



Glutaminylcystine



Cystinylglutamine

These compounds, synthesized by Harington and Mead,[36] differ from insulin in that they exert no effect on the blood sugar.

The physiological activity of insulin also seems to depend on the tyrosine. Peptic digestion of crystalline insulin results in the loss of tyrosine units and a corresponding reduction in activity.[37] It has also been shown that iodine substitution products of insulin, in which the iodine occupies the 3′, 5′ positions in the tyrosine units, are physiologically inert. Removal of the iodine causes the reactivation of the hormone.[38]

Standardization. To insure uniform potency of commercial preparations and to avoid the grave dangers of overdosage, great care is exercised in the standardization of insulin. It was recognized early that the rabbit is a suitable animal (mice are now also used) for purposes of insulin assay, and, therefore, at the first meeting of the Standardization Committee of the League of Nations, it was decided provisionally to define a unit of insulin as one-third of the amount required to lower the blood sugar of a normal rabbit, weighing 2 kg. and previously fasted for twenty-four hours, to the convulsive level (0.045 per cent) within three hours. At the present time the Health Committee of the League keeps under its auspices a preparation of insulin hydrochloride which serves as an international standard.[38a] One milligram of this standard is equivalent to 8 units. Accordingly, the definition of the international unit is the quantity (of a given preparation) which produces an effect on carbohydrate metabolism equal to that of one-eighth of a milligram of the standard preparation of insulin hydrochloride. By this definition, the mode of assay is not prescribed. It may be mentioned here that 1 mg. of crystalline insulin (as prepared by Abel and others) is equivalent to 22 international units.[39]

Action. The administration of insulin by mouth is without influence. Insulin is effective when given subcutaneously, but produces its maximum and most rapid effect when injected intravenously. The

[36] *Biochem. J.*, **30**, 1598 (1936).

[37] A. M. Fisher and D. A. Scott, *J. Biol. Chem.*, **106**, 289 (1934).

[38] C. R. Harington and A. Neuberger, *Biochem. J.*, **30**, 809 (1936).

[38a] Note: The old standard insulin has been recently discarded; the newly adopted international standard is crystalline insulin, taken as 22 units per milligram.

[39] It is essential not to neglect the factor of animal variability in bio-assay. This question has been particularly well analyzed by J. W. Trevan, *Proc. Roy. Soc.* (*London*), *B*, **101**, 483 (1927). See also, Bur. Standards Research Paper 263.

introduction of insulin into the duodenum, when the stomach is not digesting, is said to diminish hyperglycemia and glycosuria in diabetic animals. Insulin increases the storage of glycogen in the liver and promotes its utilization in the tissues, the latter being evidenced by an elevation in the respiratory quotient (p. 515). Accordingly, the injection of this hormone is followed by a rapid fall in blood sugar, and if a sufficient quantity is given, marked hypoglycemia develops. When the blood-sugar level is reduced to about 0.04 per cent, characteristic convulsions develop, particularly in the rabbit. If these symptoms are not relieved at once the animal dies. The amount of reserve glycogen is a factor, and where this is present in abundance, convulsions do not develop quite as readily as in poorly nourished animals. Insulin convulsions may be relieved by injecting glucose, and to a less extent by mannose, galactose, levulose, and maltose, but not by the pentoses xylose and arabinose nor by the disaccharides sucrose and lactose. In sheep, it may be noted, even very large doses of insulin do not cause any convulsions or other distress. The blood sugar may be depressed to about 30 mg. per 100 cc. and remain at that level for hours. In normal persons with the usual glycogen reserve, the administration of sufficient insulin may cause almost complete disappearance of blood sugar without causing convulsions. It is only when the concentration in the cerebrospinal fluid becomes similarly depressed that this symptom appears.

In the diabetic individual, insulin relieves at once the symptoms of hyperglycemia, glycosuria, acetonuria, and acidosis. There is a marked improvement in the utilization of carbohydrates, as determined by an elevation of the respiratory quotient, and in the deposition of glycogen in the liver. With these changes there is also an improvement in fat metabolism and in the conservation of the tissue proteins. Through the continued and regulated use of insulin, patients with diabetes may be maintained indefinitely in a more or less normal metabolic and nutritional state.

Modification of the Action of Insulin by Other Internal Secretions. Whereas insulin depresses the blood sugar, certain other internal secretions exert a marked opposing effect. The better known of the antagonistic secretions are epinephrine of the adrenal, thyroxine of the thyroid, and the diabetogenic substance of the anterior pituitary (p. 505). Extracts of the posterior lobe of the pituitary have also been found to have an effect in raising the blood sugar, and there is some evidence that even the adrenal cortex may participate in the maintenance of the blood-glucose level.

The diabetogenic substance of the hypophysis has received considerable attention since the remarkable demonstration by Houssay that in depancreatized dogs the symptoms of diabetes (glycosuria, hyperglycemia, acidosis) are dramatically ameliorated following extirpation of the hypophysis. The Houssay dog, by which is meant a completely depancreatized and hypophysectomized animal, may live for

many months without treatment. However, the diabetes is not completely abolished and the dog eventually dies from the accompanying cachectic condition.

Injection of extracts of the anterior lobe of the hypophysis causes glycosuria and hyperglycemia. When the gland is extirpated the action of insulin is unopposed and the blood sugar is therefore markedly lowered. This occurs also in the diabetic animal. Hypophysectomized animals and depancreatized-hypophysectomized animals (Houssay dog) are very sensitive to insulin. Contrariwise, hyperfunction of the anterior pituitary should increase the tolerance to insulin. The influence of the pituitary and adrenal glands upon pancreatic diabetes has been reviewed critically by Long.[40]

Adrenal insufficiency is often associated with a low blood sugar and a marked tendency to develop hypoglycemia following the administration of carbohydrate. The explanation is on the basis of deficient secretion of epinephrine (and cortical hormone?) and the consequent unopposed action of insulin liberated in response to the carbohydrate administered.

The thyroid secretion plays a less conspicuous rôle as an antagonist to insulin than either the adrenal or pituitary secretion.

Modification of the Action of Insulin by Protamine. Mention has been made of one of the difficulties encountered in the use of insulin, namely the possibility of the development of hypoglycemia. Owing to its quick action, insulin produces a rapid fall in blood sugar. The effect is soon dissipated, however, necessitating frequent repetition of the insulin injection, especially in the more severe diabetics. Even under ideal conditions of treatment it is practically impossible to duplicate in such patients the normal mechanism of blood-sugar regulation, such as occurs ordinarily through the continuous secretion of the hormone by the pancreas. This is revealed by the marked fluctuations which may occur. A severe diabetic in the course of a day may have blood sugar concentrations varying, for example, from 50 to 450 mg., which means that he suffers intermittently from underdosage and overdosage of the hormone.

These difficulties may now be obviated by the use of insulin combined with protamine (Hagedorn),[41] or with protamine and zinc (Fisher and Scott).[42] Combined in either form, the insulin after injection is liberated at a much slower rate, making possible a more satisfactory control of the glycemic level. Of the two preparations, the one containing zinc exerts the more prolonged effect. Because of this, longer intervals may be allowed between injections than are possible with free insulin. There are, however, circumstances in which

[40] C. N. H. Long, *Medicine*, **16**, 215 (1937).

[41] H. C. Hagedorn, B. N. Jensen, *et. al.*, *J. Am. Med. Assoc.*, **106**, 177 (1936).

[42] *J. Pharm. Exptl. Therap.*, **58**, 78 (1936). For a description of insulin-zinc-protamine, see *J. Am. Med. Assoc.*, **108**, 640 (1937).

the use of free insulin is to be preferred, as when a quick effect is desired. In other circumstances, the use of free insulin is combined with the administration of insulin-zinc-protamine, while in still other cases the treatment is restricted to the use of the latter preparation.[43]

Hyperinsulinism. The more familiar manifestation of abnormal pancreatic function is deficient secretion of insulin (*hypoinsulinism*), this being the underlying factor of the symptoms of diabetes, such as hyperglycemia, impaired utilization of carbohydrate, etc. Since the discovery of insulin, the results of experimental and therapeutic overdosage with this hormone have also received considerable attention. The outstanding effect of the presence of an excessive amount of insulin is hypoglycemia, which is accompanied by a train of symptoms, showing, in man, considerable variation. At first there is usually a feeling of nervousness or tremulousness; sometimes there is a feeling of hunger. This is followed by weakness and a sense of depression, and later a cold perspiration breaks out; frequently there is an increase in the pulse rate. Extreme anxiety, sometimes excitement and emotional instability, confusion and delirium become evident, followed ultimately by collapse. Convulsions do not occur in man, according to the description given by Fletcher and Campbell.[44] However, others have observed convulsions as an accompaniment of insulin hypoglycemia and especially of hypoglycorrhachia (low cerebrospinal fluid sugar).

Through a better understanding of the action of insulin clinical cases of *hyperinsulinism* have been more readily recognized[45] and more effectively studied. Most important of the conditions associated with spontaneous hypoglycemia, due apparently to an increased production of insulin, is malignancy of the pancreas with proliferation of the islet tissue. The first case of this type to be thoroughly studied was one of carcinoma of the islands of Langerhans with metastases in the liver and lymph nodes, described by Wilder, Allan, Power, and Robertson.[46] Their patient experienced frequent attacks of extreme weakness, faintness and paresthesia, accompanied by *hypoglycemia.* These symptoms could be relieved by eating between meals or taking sweet drinks. When food was withheld for 3 hours and 20 minutes after the noon meal, the blood-sugar concentration fell to 0.055 per cent. At this time the patient appeared apprehensive and depressed. Fifteen minutes later perspiration and tremor were noted; at 4 hours the blood sugar had fallen to 0.036 per cent and 15 minutes later to 0.027 per cent, at which time the patient was stuporous and no longer able to speak

[43] The clinical aspects of the subject have been recently discussed by R. G. Sprague and collaborators, *J. Am. Med. Assoc.*, **106**, 1701 (1936), and by E. P. Joslin, *ibid.*, **109**, 497 (1937).

[44] *J. Metab. Research*, **2**, 637 (1922).

[45] S. Harris, *J. Am. Med. Assoc.*, **83**, 729 (1924); *Endocrinology*, **16**, 29 (1932); *J. Am. Med. Assoc.*, **101**, 1958 (1933).

[46] *J. Am. Med. Assoc.*, **89**, 348 (1927).

and was jerking about convulsively. At this point 15 grams of glucose were given by mouth; the blood sugar rose to 0.065 per cent and the patient became rational and able to converse. Hourly doses of glucose were required to prevent the patient from developing too severe an hypoglycemia.

The conditions just described (overdosage with insulin, tumor of the pancreas) are examples of " true " hyperinsulinism. To these may be added the following: compensatory, or pathological, hypertrophy or hyperplasia of the islands of Langerhans; physiological hyperactivity of these structures induced by excessive carbohydrate intake.

There are, however, other circumstances under which hypoglycemia develops that are unrelated to increased insulin liberation. To illustrate, there may be adrenal insufficiency; the effect of insulin is not adequately antagonized, owing to a lack of epinephrine. The result may be severe hypoglycemia. In the absence of the normal opposing forces, the effect of insulin is exaggerated; hence the designation of " relative " hyperinsulinism.[47]

The Thyroid Gland. In man, the thyroid gland is a bilobed, reddish yellow, highly vascular organ, surrounded by a capsule of connective tissue; it weighs on the average about 20 or 25 grams and is situated at the sides of the larynx and trachea. Histologically, the organ appears to be composed of numerous closed alveoli or vesicles containing a single layer of cuboidal epithelium and filled with translucent material known as *colloid.*

Baumann,[48] in 1895, made the important discovery that the thyroid gland of mammals contains iodine in firm organic combination. On acid hydrolysis of the thyroid, he obtained an iodine compound which was named *iodothyrine.* Oswald [49] studied the colloid material of the thyroid gland and thought it to be mainly globulin. He found, moreover, that in general the amount of iodine varied with the amount of visible colloid, although hyperplastic thyroids could be rich in globulin and yet be iodine-free. It was Oswald who introduced the term *iodothyreoglobuline* for the globulin-iodothyrine complex. This is now more commonly designated as *thyroglobulin.*

Thyroxine. The isolation of the active principle of the thyroid gland was reported by Kendall[50] in 1916. From 3 tons of the fresh organ he obtained 33 grams of a substance which had the same pharmacological properties as whole thyroid gland. About 10 years later,

[47] There are still other causes of hypoglycemia which cannot be considered in the present discussion. For further information, the reader is referred to the following: S. Harris, *Ann. Internal Med.*, **7**, 1084 (1934); G. M. Wauchope, *Quart. J. Med.*, **2**, 117 (1933); A. F. Hartman and J. C. Jaudon, *J. Ped.*, **11**, 1 (1937).

[48] *Z. physiol. Chem.*, **21**, 319, 481 (1896).

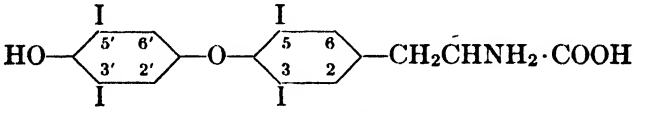
[49] *Ibid.*, **23**, 265 (1897); **32**, 121 (1901).

[50] Collected papers of the Mayo Clinic, **8**, 513 (1916); *J. Biol. Chem.*, **40**, 265 (1919); see also *Ann. Clin. Med.*, **1**, 256 (1923).

Harington [51] improved the method of isolation and obtained yields as high as 0.027 per cent from fresh gland and 0.12 per cent from dried thyroid gland. The chemical constitution of thyroxine was determined by Harington soon after; the following year, he and Barger described its synthesis.[52]

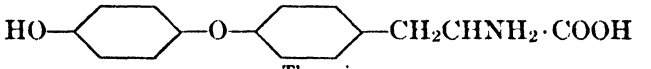
Thyroxine is *β*-3 : 5-*Diiodo*-4-(3′ : 5′-*diiodo*-4′-*hydroxyphenoxy*) *phenyl* -*α*-*aminopropionic acid.*

A simpler nomenclature is 3 : 5, 3′ : 5′-tetraiodothyronine.



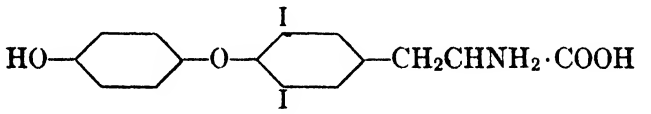
Thyroxine
3 : 5 , 3′,5′-Tetraiodothyronine

Thyronine is formed from thyroxine by catalytic deiodination.



Thyronine

Loss of only two of the iodine atoms yields 3 : 5-*diiodothyronine.* This compound is of interest because it possesses the physiological activity characteristic of thyroxine, though naturally in a lesser degree.



3 5-Diiodothyronine

Harington [53] has resolved the racemic form, *dl*-thyroxine, into its two optically active isomers. These have also been prepared by first resolving *dl*-3 : 5-diiodothyronine, then iodinating at the 3′, 5′ positions. *l*-Thyroxine has also been obtained from thyroid gland by the action of proteolytic enzymes.[54]

Diiodotyrosine. Only a part of the organic iodine contained in the thyroid is in the form of thyroxine.[55] Another iodine derivative of tyrosine is present, namely 3,5-diiodotyrosine, discovered independently by Harington and Randall [56] and by Foster.[57] From a sample of 100 grams of partially purified thyroglobulin, containing 760 mg. of iodine,

[51] *Biochem. J.*, **20**, 293, 300 (1926).

[52] *Ibid.*, **21**, 169 (1927); a footnote in the paper by Harington and Barger states that the chemical constitution of thyroxine was discovered independently by Dakin.

[53] *Ibid.*, **22**, 1429 (1928); J. H. Gaddum, *ibid.*, **22**, 1434 (1928).

[54] C. R. Harington and W. T. Salter, *Biochem. J.*, **24**, 456 (1930).

[55] J. P. Leland and G. L. Foster, *J. Biol. Chem.*, **95**, 165 (1931); see also N. F. Blau, *ibid.*, **102**, 269 (1933).

[56] *Biochem. J.*, **23**, 373 (1929).

[57] *J. Biol. Chem.*, **83**, 345 (1929).

Foster isolated 0.44 gram of diiodotyrosine, containing 248 mg. of iodine. This accounted for 33 per cent of the total iodine. The thyroxine which was isolated accounted for an additional 16 per cent of iodine. These observations indicate that thyroglobulin may contain still other iodine compounds and also that 3,5-diiodotyrosine may be the precursor of thyroxine. It is to be noted, however, that this derivative of tyrosine is without the physiological properties possessed by thyroxine.[58]

3 : 5-Diiodotyrosine

Summary of the Physiological Action of the Thyroid Gland. The thyroid plays a very fundamental rôle in controlling the oxidative processes of the organism, the effect being generalized, rather than restricted to a few tissues. If an excess of the active principle is present, metabolism is augmented; if there is a deficiency, metabolism is depressed. Stimulation of cellular oxidation by thyroid apparently involves an increase in the utilization of carbohydrate, fat, and nitrogenous metabolites. It is obvious that, corresponding to the increased combustion of these substances, there would be a rise in oxygen consumption and carbon dioxide production. Accordingly, measurement of the gaseous exchange and comparison with normal data provides information concerning the rate of total metabolism and, indirectly, of the amount of heat produced.

Another function is heat regulation. It is assumed that normally the thyroid controls metabolism at a fairly constant rate, but that if the need for increased cellular oxidation arises, as when an animal is exposed to cold, the gland is stimulated to increased activity. As a result, heat regulation is maintained. But, if the animal suffers from thyroid insufficiency, heat regulation is deficient; indeed, thyroidectomized animals are unable to withstand prolonged exposure to severe cold.

The thyroid is also of importance in relation to growth and differentiation, as is illustrated by the fact that the metamorphosis of tadpoles into frogs is dependent on thyroid secretion; if the gland is removed, this change does not take place, although the tadpole may continue to grow, as such. If at any time thyroid is given, prompt metamorphosis occurs. Gudernatsch [59] discovered that the feeding of thyroid to young tadpoles results in premature metamorphosis with the formation of exceedingly small frogs, often no larger than a fly. Traces

[58] 3,5-Diiodotyrosine is widely distributed in certain marine organisms. It was first discovered by Drechsel (*Z. Biol.*, **33**, 85 [1896]) among the hydrolytic products of the axial skeleton of the Gorgonian coral. Mörner (*Z. physiol. Chem.*, **51**, 33 [1907]; **55**, 77, 223 [1908]) found it in the skeleton of certain Anthozoa, and Wheeler and Mendel (*J. Biol. Chem.*, **7**, 1 [1909]) in sponges.

[59] *Zentr. Physiol.*, **26**, 323 (1912).

of iodine may exert a similar effect, according to Swingle.[60] A species of salamander, found in Mexico, never undergoes metamorphosis, apparently because of the absence of the thyroid. Metamorphosis can be induced artificially, however, by thyroid feeding.

Physiological Activity of Various Compounds Associated with the Thyroid Gland and Related to Thyroxine. One of the methods that has proved of some value in estimating the potency of thyroid substance is based on the effect on the basal metabolic rate. The usefulness of the method is enhanced by the fact that it may be applied both to human subjects and experimental animals. Another biological method of assay depends on the acceleration of metamorphosis in Amphibia; a third method has been based on the observation of Hunt that in mice the administration of active thyroid substance antagonizes the toxic effect of actonitrile.

On empirical grounds the iodine content has been used as a basis of standardizing thyroid preparations. After the isolation of thyroxine, it seemed inconsistent that the total iodine could represent the potency of different preparations, since the thyroxine is equivalent to only a part, and a variable part, of the total iodine, and especially since the other important iodine compound, diiodotyrosine, is physiologically inert. Accordingly a chemical method for assaying thyroid on the basis of the thyroxine content was devised. It was anticipated that the physiological activities of various preparations of thyroid would parallel their thyroxine-iodine contents, but this has not been the experience of all workers. Indeed, Means and associates[61] have found the physiological activity to be more definitely related to the total iodine content, which if correct must signify that diiodotyrosine is present in the gland in a form which is physiologically perhaps as active as thyroxine. Before pursuing this point, consideration should be given to contradictory evidence submitted by others and to other points concerning which there seems to be difference of opinion.

First it may be stated that the results on the relative effects of *d*- and *l*-thyroxine have not been uniform. Gaddum,[62] investigating the problem, found that *l*-thyroxine was about three times as effective as *d*-thyroxine in raising the oxygen consumption of rats. Salter[63] compared the calorigenic action of the two isomers in patients with myxedema and could determine no difference in potency. On the other hand, Foster, Palmer, and Leland[64] found that *l*-thyroxine exerts a calorigenic effect (based on oxygen consumption of guinea pigs) approximately twice as great as that of *dl*-thyroxine. If the last observation is correct, it follows that *d*-thyroxine is physiologically inert.

[60] *J. Gen. Physiol.*, **1**, 593 (1919).

[61] J. H. Means, J. Lerman, and W. T. Salter, *J. Clin. Investigation*, **12**, 683 (1933); W. T. Salter and J. Lerman, *ibid.*, **14**, 691 (1935).

[62] *J. Physiol.*, **68**, 383 (1929–30).

[63] *J. Clin. Investigation*, **14**, 37 (1935).

[64] *J. Biol. Chem.*, **115**, 467 (1936).

It seems to be fairly generally conceded that, on the basis of iodine equivalents, thyroglobulin is physiologically more active than thyroxine, but there is no agreement as to the explanation for this discrepancy. Indeed, attempts to correlate the action of thyroid substance and thyroglobulin with the thyroxine content have yielded divergent results. Thus Palmer and Leland [65] have reported that the calorigenic action of thyroid substance from various sources (normal and pathological human glands, commercial desiccated thyroid preparations, hog thyroglobulin) was definitely proportional to the thyroxine content and not to the total iodine. This is at variance with the results of others (Means, Lerman, and Salter [61]) who found the calorigenic effect related, not to thyroxine, but to total iodine.

Diiodotyrosine-peptone, obtained from thyroglobulin by controlled digestion with pepsin, is physiologically inert. But if this inactive fraction is concentrated and then treated with pepsin, a procedure designed to further peptic synthesis, the product, an iodine-containing substance of high molecular weight, is found to resemble natural thyroglobulin in its chemical and biological properties and is capable of ameliorating the symptoms of human myxedema (Salter and Pearson,[66] Salter and Lerman [67]). If this remarkable observation is confirmed and its significance elucidated, it may lead to a revision of our present conception of the hormone of the thyroid gland.

From the foregoing considerations it is seen that our understanding of the chemistry of the active principle of the thyroid gland is incomplete and at present unsettled. It is of interest, however, that the physiological properties of thyroxine are retained in a measure in certain of its derivatives. For example, 3 : 5-diiodothyronine in appropriate doses produces a rise in metabolism and is therapeutically active. It has also been stated that the bromine analogue of thyroxine, 3 : 5, 3′: 5′-tetrabromothyronine, exhibits the characteristic physiological activity of thyroxine although naturally in a very much lower degree (Harington [68]).

Diseases of the Thyroid Gland. The manifestations of deranged thyroid function are exceedingly variable; nevertheless, most symptoms may be related to either diminished function (hypofunction) or increased function (hyperfunction) of the gland.

Myxedema first described by Gull in 1874, is a manifestation of hypothyroidism. This condition results from atrophy of the thyroid gland and the consequent reduction in hormone secreted. It may be produced also by partial or complete extirpation of the gland. Inasmuch as the hormone regulates cellular oxidation, its deficiency results in a marked reduction of the basal metabolic rate (see next chapter). The temperature of the body and the pulse rate tend to be subnormal.

[65] *J. Clin. Investigation*, **14**, 619 (1935).
[66] *J. Biol. Chem.*, **112**, 579 (1936).
[67] *Endocrinology*, **20**, 801 (1936).
[68] *Ergebnisse Physiol.*, **37**, 210 (1935).

Chilliness, even when it is warm, and sensitiveness to cold are characteristic features and reflect a deficient temperature-regulating mechanism. The face and hands become puffed and swollen. This is not due to edema, but to thickening of the subcutaneous connective tissue. The skin is thick and has an unhealthy appearance, and the hair tends to fall out. Myxedematous individuals are sluggish both mentally and physically, are often anemic, and tend to be obese, although this is not always the case.

These symptoms, which profoundly affect the personality of the afflicted individual, are usually ameliorated by feeding thyroid gland or extracts of it, or by the administration of thyroxine. The effects of the administered thyroid hormone may last for several weeks. However, when treatment is discontinued for longer periods there is a relapse, showing that the active principle of the thyroid gland is not stored indefinitely.

It is of interest that the blood cholesterol is conspicuously elevated in myxedema, values over 500 mg. per 100 cc. being relatively common. Effective therapy causes a reduction.

Cretinism. Failure in the embryonic development of the thyroid or its atrophy during fetal life or childhood results in the condition which is commonly called cretinism, but which would be more accurately designated by the term *infantile* or *childhood myxedema*. There are two forms of this disease. The endemic form is due presumably to goitrous degeneration of the gland and is found in districts where goiter is endemic. The failure in thyroid development in this form may be due to a lack of iodine in the organism of the mother. The sporadic form of cretinism may occur anywhere. As to its etiology, nothing is known except that it may have the same underlying causes as myxedema.

The most noticeable symptom is the practically complete cessation of physical and mental development, resulting in dwarfism and idiocy. Cretins are typically pot-bellied, ugly, and somewhat obese. The hair is thick and coarse, and the skin dry and pale. As in myxedema of adults, the basal metabolic rate is low.

Concerning the deficiency of the thyroid hormone in this condition, there can be little question. Cretins fed on whole thyroid gland or treated with thyroxine tend to develop normally. There is almost immediate improvement both mentally and physically; an ugly, idiotic child may be converted into an almost normal one. The cures which have been accomplished in this way are most remarkable. However, in the case of cretinism of long standing, treatment is not as successful.

Creatinuria, which occurs normally in infancy and childhood, is said to be diminished or absent in cretinism. In untreated cases of cretinism, as in untreated myxedema, the blood cholesterol is markedly elevated.

Endemic or Colloid Goiter. Hyperplasia or enlargement of the thyroid does not necessarily indicate hyperfunction and may be due fundamen-

tally, as in this case, to a deficiency in the hormone mechanism. Being easily recognized by the marked swelling which develops in the region of the neck, this disease was known to the ancients. The enlargement is due to an increase in the colloid material of the gland, hence the name " colloid goiter."

Goiter is endemic in many parts of the world, but is especially prevalent in certain sections of Switzerland and in the region of the Great Lakes in the United States. That it is due to a lack of iodine was shown by Marine and Kimball.[69]

McClendon [70] has reported the results of analyses of drinking water obtained from regions where goiter is endemic and from other regions. These show higher values for iodine in the water from non-goitrous regions than in that from regions where goiter is prevalent. Not only the water but also the soil and hence the vegetation in such areas are low in iodine. Because of this deficiency, the normal formation of thyroxine and thyroglobulin is limited, and it has therefore been surmised that in an attempt to compensate for the poorness in the quality of the secretion, the gland is stimulated to hyperactivity and the production of excessive amounts of colloid. That not infrequently the metabolism of patients with endemic goiter is approximately normal is evidence that the hyperplasia of the gland may compensate more or less for the deficiency. Just as often, however, the basal metabolic rate tends to be low, indicating that, despite overactivity, which is sometimes very pronounced, sufficient thyroxine for the needs of the organism is not being produced.

Recognition by Marine and associates of the etiological relationship of iodine deficiency to endemic goiter led them to suggest as the method of treatment the administration of small doses of iodides. Prophylactic measures are now taken in many goiter regions by adding simple inorganic iodides to the drinking water supply, and to the table salt, and by periodically administering therapeutic doses of iodide to school children. This procedure is to be especially recommended in the case of adolescent girls and during pregnancy.

Simple goiter is so prevalent in the Great Lakes basin that a large proportion of the dogs and other animals of the region are afflicted with it. The practice of administering small amounts of iodide to stock animals for the purpose of preventing goiter has yielded remarkably beneficial results.

Goiter may be produced in rabbits by prolonged feeding of cabbage from certain localities.[71] A search for the goitrogenic factor involved,

[69] *J. Am. Med. Assoc.*, 77, 1068 (1921); D. Marine, "The Functions of the Thyroid Gland," *Physiol. Rev.*, 2, 521 (1922).

[70] *J. Am. Med. Assoc.*, 82, 1669 (1924); see also the review "The Distribution of Iodine with Special Reference to Goiter," *Physiol. Rev.*, 7, 189 (1927).

[71] A. M. Chesney, T. A. Clawson, and B. Webster, *Bull. Johns Hopkins Hosp.*, 43, 261 (1928); Webster, *Endocrinology*, 16, 617 (1932).

by Marine and associates,[72] has revealed it to be cyanide. Further experiments have shown that substances which depress oxygen consumption may increase thyroid activity and that cyanides are among the most potent of these goitrogenic agents. Accordingly, the view has been expressed that a deficiency of iodine, though certainly the immediate cause of thyroid hyperplasia, is in most cases only relative, and is due to the increased demands for iodine caused by a goitrogenic agent.

Exophthalmic goiter (also known as *Graves'* and as *Basedow's disease*) is the most important example of hyperthyroidism. An increased basal metabolic rate is the most prominent finding in this condition. The temperature of the body is usually above normal; the heart rate is faster than normal and is irregular. There is usually an enlargement of the thyroid gland. Among other symptoms are a marked tendency to emaciation, restlessness, hyperexcitability, and gastrointestinal disturbances. Usually, though not always, there is protrusion of the eyeball (" exophthalmos ").

Most physiologists believe exophthalmic goiter to be due to hypersecretion of the thyroid hormone, in view of the fact that some of the symptoms of this disease may be produced by the administration of sufficient doses of thyroxine. Another point in support of this general idea is that many of the symptoms of exophthalmic goiter are exactly the reverse of those noted in myxedema. Since the disease is presumably due to excessive secretion, the methods of treatment consist in diminishing the amount of active thyroid by partial extirpation of the gland, by ligation of the thyroid arteries, or by exposure to Roentgen rays or radium.

The view has been advanced that simple goiter, which is essentially a condition of hypothyroidism, may pass over into Graves' disease.

A study of the iodine partition in the thyroid in clinical hyperthyroidism has disclosed that in untreated cases the gland is relatively low with respect to its thyroxine content, this doubtless representing a depleted or exhausted state of the gland.[73] However, the administration of iodine (this being an accepted method of temporarily treating cases of exophthalmic goiter and thyroid adenoma) results in a marked increase of both the inorganic- and thyroglobulin-iodine fractions. A definite change in the chemical nature of the thyroglobulin is indicated by the relative and absolute increases in the thyroxine fraction and a consequent relative, though not absolute, decrease in the percentage of thyroglobulin iodine present as diiodotyrosine.

Relation of the Thyroid to Other Glands. Probably no gland of internal secretion acts independently of the others. There is abundant

[72] D. Marine, E. J. Baumann, A. W. Spence, and A. Cipra, *Proc. Soc. Exptl. Biol. Med.*, **29**, 772, 822 (1931–32).

[73] A. B. Gutman, E. M. Benedict, B. Baxter, and W. W. Palmer, *J. Biol. Chem.*, **97**, 303 (1932).

evidence that the function of the thyroid is probably closely interrelated with the activity of the pancreas, adrenals, pituitary, thymus, and gonads. Some of these interrelationships have not been clearly defined; others seem more explicit. The relation of the thyroid to the pancreas may be exemplified by the increased sensitivity of the thyroidectomized animal to the hypoglycemic action of insulin and the resistance to insulin which may be induced by the administration of thyroid or thyroxine to such thyroid-deficient animals. With regard to the adrenals, it is supposed that the cortex exerts an inhibitory effect on the thyroid. It is pointed out that involution of the adrenal cortex in the new-born is accompanied by stimulation of the thyroid, this coinciding with the increased heat production which begins a few days after birth. Marine has advanced the view that the disease of the adrenal cortex may be the primary factor in the etiology of hyperthyroidism. This hypothesis stands in need of fuller confirmation.

The thymus seems to exert an antagonistic effect, for when it is fed to tadpoles, metamorphosis is delayed. This may be counterbalanced by feeding thyroid. Thyroidectomy is said to hasten thymus involution, while feeding thyroid seems to exert the opposite effect. It is probable that the gonads and thyroid are functionally interrelated, either directly, or through the mediation of the pituitary, although much of the available information is rather confusing and somewhat difficult to interpret.

Of the various interrelationships the one that is perhaps most clearly recognized is that of the anterior pituitary which elaborates a principle that has been described as the thyrotropic hormone. This has the specific property of stimulating the thyroid gland, causing it to hypertrophy. In hypophysectomized animals, the thyroid undergoes atrophy. The administration of the thyrotropic hormone, in sufficient amounts, produces essentially all the symptoms associated with hyperthyroidism. There is therefore the possibility that the anterior pituitary may be a factor in the etiology of Graves' disease, but further evidence is needed before this speculation can be justified. The thyrotropic hormone will be referred to later in another connection (p. 504).

In a considerable proportion of persons with hyperthyroidism the blood iodine is conspicuously elevated. Perkin and Cattell [74] have reported the following values, expressed in micrograms per 100 cc. of blood:

	Range of Values	Average
Normal	1 5–12	6.8
Non-toxic goiters	1 6–12 8	7.0
Hyperthyroidism	2–155	21.0

Urinary excretion of iodine is increased in hyperthyroidism (Puppel and Curtis).[75]

[74] *N. Y. State J. Med.*, **36**, 1033 (1936).
[75] *Arch. Internal Med.*, **58**, 957 (1936).

Mention has been made of the high blood cholesterol in myxedema and cretinism. In severe hyperthyroidism, the cholesterol is often markedly diminished, values of approximately 60 mg. per cent being fairly common.

The Parathyroid Glands. The earlier physiologists and surgeons had observed that thyroidectomy frequently led to the development of tetany, followed by death. That this outcome was actually due to the accidental removal of an independent set of glands was not generally appreciated until the beginning of the present century, despite the fact that the parathyroid glands had been discovered twenty years previously (1880) by the Danish anatomist Sandstrom.[76] There are usually two pairs of parathyroid glands, one pair lying on each side of the trachea, close to the thyroid or embedded in it. At least in some animals (cat, rabbit, etc.) there are probably additional or accessory parathyroid structures distributed along the trachea near by. The parathyroids are small glands, yellowish brown to brown-red in color, usually bean-shaped in structure, and about the size of a hemp-seed or somewhat larger; in man they are variable in length (3–15 mm.), about 2–3 mm. in breadth and 2 mm. in thickness.

In 1925, Hanson [77] and Collip,[78] working independently, succeeded in preparing an active and relatively pure extract from the parathyroid gland. The hormone preparation, named parathormone, exhibited a marked effect in raising the calcium concentration of the blood both in normal and parathyroidectomized animals. The work of Collip and others has contributed toward establishing the theory that the parathyroid glands secrete a hormone which is concerned with the regulation of calcium and phosphorus metabolism and with controlling, in some way, the concentrations of calcium and inorganic phosphate in the blood. As yet, the chemical nature of the parathyroid hormone has not been determined. Tweedy's work [79] indicates that, like insulin, it is probably in the nature of a protein.

Hypoparathyroidism. The extirpation of the parathyroids in man and most animals, particularly the carnivora, results in *tetany.*[80] The dog is especially susceptible. Usually in from one to four days after the operation, symptoms of intoxication become manifest. There is

[76] *Upsala läkerför. forh.*, **15**, 44 (1880).

[77] *Military Surgeon*, **52**, 434 (1923); **54**, 76, 218, 554 (1924); *Proc. Soc. Exp. Biol. Med.*, **22**, 560 (1925).

[78] *J. Biol. Chem.*, **63**, 395 (1925).

A review on the subject of the parathyroid glands has been prepared by D. L. Thomson and J. B. Collip, Physiol. Rev., **12**, 309–383 (1932). Work which has appeared since the preparation of this review has been summarized by these authors in *Ann. Rev. Biochem.*, **2**, 242 (1933). The reader is also referred to the monograph by D. H. Shelling, "The Parathyroids in Health and Disease," St. Louis, 1935.

[79] *J. Biol. Chem.*, **99**, 155 (1932–33); **108**, 105 (1935); **116**, 163 (1936).

[80] Parathyroid insufficiency as the cause of tetany was established by W. G. MacCallum and C. Voegtlin, *J. Exptl. Med.*, **11**, 118 (1909).

loss of appetite, the motor nerves become hyperexcitable to electrical but not to mechanical stimuli, and there is marked restlessness. Diarrhea, often bloody, is a frequent symptom. Soon fine tremors set in, and the animal gradually becomes stiff. The tremors become more and more violent, the temperature, respiration, and heart action increase. After nine or ten days the animal dies in spasm or convulsions, or from exhaustion.[81] The effects of loss of parathyroid function are especially severe where the calcium requirement is increased as in pregnant or lactating animals, or in animals with active rickets. In parathyroid tetany, the outstanding change in the composition of the blood is a marked decrease in the concentration of calcium. Normally human and dog serum contains 10 to 11 mg. of calcium per 100 cc. Following the removal of the parathyroid, the concentration may fall to 5 to 6 mg., and even lower. The concentration of calcium ions is particularly affected. The symptoms may be relieved at this time by the administration of calcium salts, such as calcium lactate, the disappearance of the symptoms being associated with an increased concentration of calcium and especially of the calcium ions in the blood. However, at least in certain animals, the administration of parathyroid extract is much more effective, especially if administered together with calcium lactate. The use of parathyroid hormone together with calcium clinically has, on the whole, yielded satisfactory results.

In parathyroid deficiency, the low serum calcium is accompanied by a high serum inorganic phosphate. According to Jones,[82] if the latter is kept low (the addition of 4 per cent basic aluminum acetate to the diet produces this effect in rats), the calcium is not depressed and tetany does not appear.

Tetany may occur spontaneously, especially in infants and children, and as in parathyroidectomized animals is associated with a low serum calcium. When it cannot be ascribed to known causative factors, such as dietary deficiency with respect to calcium and vitamin D, the condition is usually designated as *idiopathic tetany*. Inasmuch as relief from symptoms has been obtained in some instances through the administration of parathyroid hormone, the presumption is that it may represent a form of depressed parathyroid function, or *hypoparathyroidism*.[83]

Hyperparathyroidism. Collip found that the administration of parathyroid hormone to dogs resulted in a sharp rise of the serum calcium to 15 mg. per 100 cc., and higher, an effect quite the opposite from that associated with diminished or absent parathyroid function.

Just as the intensive study of insulin ultimately focused attention

[81] For a detailed description of the symptoms of parathyroid tetany consult L. R. Dragstedt, *Physiol. Rev.*, 7, 499 (1927).

[82] *J. Biol. Chem.*, 115, 371 (1936).

[83] The subject of hypoparathyroidism, in its clinical aspects, has been reviewed by R. H. Freyberg, R. L. Grant, and M. A. Robb, *J. Am. Med. Assoc.*, 107, 1769 (1936).

on the subject of pancreatic hyperfunction, or *hyperinsulinism*, so the work of Collip was doubtless a factor which attracted attention to the problem of clinical *hyperparathyroidism*.[84]

Enlargement of the parathyroid glands in patients suffering from diseases of bone, notably *osteitis cystica* (Recklinghausen's disease of bone), was noted by the pathologist Askanazy in 1904, but the significance which clinicians and others attached to this and similar observations was that the enlargement was a compensatory hypertrophy, secondary to the bone disease. However, the beneficial effects obtained by Mandl [85] twenty years later in a case of generalized osteitisfibrosa cystica by removal of a parathyroid adenoma suggested that the disease in the bone was perhaps the *result* and not the cause of the hyperactivity of the gland. Soon followed reports of other cases in which the hypercalcemia, decalcification of bone, and other changes in this disease were related to hyperfunction of the parathyroids. The concept of the etiological relationship of hyperparathyroidism to Recklinghausen's disease of bone was finally established through the demonstration by Jaffe, A. Bodansky, and Blair [86] that a similar condition may be produced experimentally in dogs and guinea pigs by the regulated administration of parathyroid hormone.

As has been stated elsewhere, the serum calcium is usually elevated in hyperparathyroidism; in one clinical case as high a value as 23.6 mg. per 100 cc. has been recorded. The inorganic phosphate tends to be low. However, these changes are not always noted. A negative calcium balance may exist and considerable amounts of this element may be excreted, without being reflected by an increased calcium content in the serum. Such findings are more likely to occur in individuals (or experimental animals) maintained on a low calcium intake. Indeed, under these conditions even hypocalcemia may develop.

Another characteristic change in hyperparathyroidism is an increase in serum (or plasma) *phosphatase*.[87] This is an enzyme, occurring in the intestinal mucosa, kidney, leukocytes, bone, cartilage, and connective tissue, which possesses the ability to liberate inorganic phosphate from organic phosphate compounds. It is also capable of reversing the reaction, thus participating in the synthesis of organic phosphates. Its full significance and the mechanism of its action in bone metabolism remain to be clarified.

Relation to Anterior Pituitary. According to Houssay,[88] pituitary insufficiency in the dog is associated with cellular atrophy of the para-

[84] The term "hyperparathyroidism" was first applied to clinical Recklinghausen disease by D. P. Barr and H. A. Bulger, *Am. J. Med. Sci.*, **179**, 440 (1930).

[85] F. Mandl, *Arch. klin. Chir.*, **143**, 1, 245 (1926).

[86] *J. Exptl. Med.*, **52**, 669 (1930); *Arch. Pathol.*, **11**, 207 (1931); *J. Biol. Chem.*, **88**, 629 (1930); *J. Exp. Med.*, **53**, 591 (1931).

[87] H. D. Kay, *J. Biol. Chem.*, **89**, 249 (1930); A. Bodansky, *ibid.*, **101**, 93 (1933); A. B. Gutman, T. L. Tyson, and E. B. Gutman, *Arch. Internal Med.*, **57**, 379 (1936).

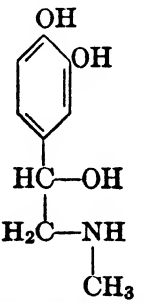
[88] *Harvey Lectures*, **31**, 116 (1935–36).

thyroids. Smith [89] has reported parathyroid atrophy after hypophysectomy in the rat. The administration of anterior pituitary extract, on the other hand, leads to proliferation of the parathyroid cells and may even cause a rise in serum calcium. The rise does not occur in the absence of the parathyroid gland. It has also been observed (Hertz and Albright [90]) that the urine of patients with hyperplasia of the parathyroids contains a substance which produces parathyroid hyperplasia when injected into rabbits. The foregoing evidence points to the controlling influence exerted by the anterior pituitary over the parathyroid. The existence of a parathyrotropic hormone has been postulated.

The Adrenals. The *adrenal* or *suprarenal* glands are two small, highly vascular organs, situated in most animals at the upper poles of the kidneys and each weighing, in man, about 6 or 7 grams. Two parts are distinguishable, the *cortex* and the *medulla*. These differ from each other embryologically, histologically, and functionally. The medulla is stained by potassium dichromate, hence it has been described as the chromaffin tissue. It is closely related to the sympathetic nervous system, developmentally and physiologically. The importance of the adrenals is indicated by the fact that their extirpation results fatally. It is now generally held that death in adrenalectomized animals is due primarily to the absence of the cortex.

However, it was from the adrenal medulla that the first hormone was isolated. It is variously called adrenaline, epinephrine, or suprarenine.

Epinephrine was first obtained as the benzoyl derivative by Abel [91] and subsequently as the free base by Aldrich [92] and Takamine.[93] It has since been prepared in the laboratory by synthetic methods. Epinephrine is closely related to tyrosine, as shown by the formula:



Epinephrine or adrenaline

[89] *J. Am. Med. Assoc.*, **88**, 158 (1927).

[90] *Proc. Assoc. Am. Physicians*, May, 1934, cited by Collip, "Glandular Physiology and Therapy," p. 115, Chicago, 1935.

[91] *Bull. Johns Hopkins Hosp.*, **9**, 215 (1898); **12**, 80 (1901).

[92] *Am. J. Physiol.*, **5**, 457 (1901).

[93] *Am. J. Pharmacy*, **73**, 523 (1901).

Before the discovery of adrenaline, Oliver and Schäfer [94] had shown that extracts of the adrenals exert a powerful effect in raising blood pressure. It has since been shown that 0.001 mg. of adrenaline, when injected into a cat, is sufficient to cause constriction of the arterioles and, hence, a rise in blood pressure. With few exceptions the effects of adrenaline on various organs and tissues are the same as those produced by stimulating the sympathetic nerve supply.[95]

From the standpoint of metabolism may be mentioned the effect of adrenaline in causing increased glycogenolysis. The immediate effect is hyperglycemia. There is also an increase in the metabolic rate as well as in the respiratory quotient, the latter change showing an increased utilization of carbohydrate. Associated with these changes is an increase in muscular power and an apparent resistance to fatigue. Opinion is divided regarding the effect of epinephrine on muscle glycogen. In rats, Cori [96] observed a decrease in glycogen accompanied by an increase in lactic acid, but no change in creatine-phosphate. Contrary results with respect to the disappearance of glycogen have been reported by

[94] *J. Physiol.*, **18**, 230 (1895).

[95] To explain the significance of adrenaline, W. B. Cannon ("Bodily Changes in Pain, Hunger, Fear and Rage," Appleton, New York and London, 1915) postulated the theory that this substance enables the organism to cope with emergencies. Cannon believes that fear, rage, and other emotions stimulate the adrenals to increased production of adrenaline, which, on entering the circulation, produces prompt mobilization of carbohydrate. This provides ready fuel for the muscles. Among the other manifestations of hypersecretion or injection of adrenaline are an increase in blood pressure and increased efficiency of muscular contraction, including that of the heart muscle. These changes Cannon believes to be adaptations which enable the organism to work at its maximum capacity in the face of danger. The effect of adrenaline in diminishing the clotting time of blood is regarded by Cannon as another adaptation, useful to the organism in preventing excessive hemorrhage in the case of wounds. See also W. B. Cannon, "The Wisdom of the Body," W. W. Norton & Co., New York, 1932.

In a well-known experiment, Cannon compared the concentration of adrenaline in the blood of normal cats with the concentration observed in the blood when the cats were frightened by the barking of a dog. Whereas, in the normal state, no evidence of adrenaline was found, the blood of the frightened animals was found to contain demonstrable amounts of this substance. These observations and conclusions have been questioned by Stewart and Rogoff. According to Stewart (*Physiol. Rev.*, **4**, 163 [1924]), the best evidence for the view that the epinephrine output exerts no important or indispensable function is that, after its suppression, the animals do not differ notably from normal animals in their blood-sugar content, in their capacity to meet the emergencies of life, or in a variety of other ways in which Cannon observed significant differences.

The existence of an epinephrine-like hormone outside the adrenal medulla and possibly in connection with the sympathetic nervous system is indicated by work from Cannon's laboratory. This substance has been called "sympathin." *Am. J. Physiol.* **96**, 377, 392 (1931).

From Szent-Gyorgyi's laboratory has come the report of the occurrence in the adrenal medulla of a hormone similar in action, but much more potent than adrenaline. *J. Physiol.*, **76**, 181 (1932). The substance has been called "novadrenin."

[96] *Am. J. Physiol.*, **94**, 557 (1930).

Soskin [97] in experiments on dogs and by Firor and Eadie [98] in the cat.

Adrenal Cortex Insufficiency; Addison's Disease. In 1855, an English physician named Addison pointed out that the peculiar and fatal disease often associated with bronzing of the skin was in some way connected with degeneration of the adrenals. This condition, now known as Addison's disease, though usually associated with tuberculosis of the adrenal glands as in the cases studied by Addison, may, however, be due to destruction of these glands by any process. The more important symptoms are pigmentation or bronzing of the skin and hair, excessive muscular weakness leading to prostration, mental depression and other nervous symptoms, gastrointestinal disturbance including vomiting, atrophy of the sex organs, and hypoglycemia. Addison's disease appears to be due to *hypofunction* of the adrenals, and it now seems to be definitely established that the more severe symptoms are the result of cortical involvement.[99]

Several years ago the results of investigations conducted in several laboratories made it seem very probable that the adrenal cortex possessed a hormone indispensable to life.[100]

In 1927 Stewart and Rogoff [101] obtained a physiologically active preparation simply by extracting dogs' adrenals with 0.9 per cent sodium chloride and glycerine. The extract was freed from adrenaline, but was otherwise relatively crude; nevertheless it proved effective in prolonging the life-span of adrenalectomized dogs. The same year Hartman and associates [102] reported the extraction of the adrenal-cortical principle from beef adrenals by means of acidulated water; the active substance was precipitated by salting out with sodium chloride and separated from protein by redissolving the hormone in 80 per cent alcohol. This preparation prolonged the lives of adrenalectomized cats. After improving the method of extraction, Hartman obtained a preparation with which adrenalectomized cats were kept alive almost indefinitely. Extraction of the cortical principle by means of organic solvents was introduced by Swingle and Pfiffner.[103]

Although this work provided strong evidence of the existence in the adrenal cortex of a hormone essential to life, more definite proof was

[97] *Ibid.*, **81**, 383 (1927).

[98] *Ibid.*, **94**, 615 (1930).

[99] For a review of the literature on adrenal insufficiency up to 1930, consult S. W. Britton, *Physiol. Rev.*, **10**, 617 (1930).

[100] F. A. Hartman, K. A. Brownell, and W. E. Hartman, *Am. J. Physiol.*, **95**, 670 (1930).

[101] *Science*, **66**, 327 (1927); *Am. J. Physiol.*, **84**, 660 (1928); **91**, 254 (1929).

[102] *Proc. Soc. Exptl. Biol.*, **25**, 69 (1927).

[103] *Anat. Rec.*, **44**, 225 (1929); *Medicine*, **11**, 371 (1932); W. W. Swingle, "The Cortical Hormone of the Adrenal Gland," *Harvey Lectures*, **27**, 33 (1931–32).

Descriptions of the various methods of extraction originally used, as well as later modifications, are given by B. Harrow and C. P. Sherwin, "The Chemistry of the Hormones," Chapter V.

needed before this could be established with certainty. Among the objectives in the further pursuit of this problem were the isolation of the hormone in pure form and the determination of its chemical constitution. Work in this direction has resulted in the isolation from adrenal-cortical extracts, not of one, but of several substances, the significance of which is as yet only partly known. It has also become more and more apparent that the effects of cortical insufficiency are many and diverse. This may mean that the adrenal cortex elaborates a hormone possessing diversified action, or that this hormone exerts a deep-seated and generalized effect which manifests itself in a variety of ways, or that there are a number of active principles, each of which is more or less specific in its action.

The known phenomena associated with adrenal-cortical insufficiency in patients with Addison's disease, as well as in experimental animals, have been described by numerous observers. A group of the manifestations seem to be related, wholly, or in part, to the changes in sodium metabolism which the adrenal cortex apparently controls. Within a few days after the removal of the adrenals, or after discontinuing injections of cortical extract, the excretion of sodium through the kidneys becomes very markedly increased. This is accompanied by a lowered concentration of sodium in the serum, which in turn affects the concentration of chloride, or bicarbonate, or both (Loeb,[104] Harrop [105]). These changes are progressive, the continued loss of salt from the tissues bringing about a loss of fluid from the body and a reduction of the circulating blood volume. Owing to the hemoconcentration, the oxygen capacity of the blood increases, as does the concentration of the plasma protein. The rate of blood flow, body temperature and basal metabolism diminish. These are often followed by a sharp fall in blood pressure.

That all these developments are related to the low level of sodium chloride is shown by the fact that they may be ameliorated by the administration of liberal amounts of salt; on the other hand, the symptoms of adrenal insufficiency are aggravated by decreasing the salt intake.

It is to be noted that serum potassium is elevated in adrenal insufficiency and may be partly responsible for the symptoms, a number of workers having shown potassium salts to be especially toxic in Addison's disease.

Adrenal cortical insufficiency is believed to cause impairment of renal function, as shown by the retention of urea, creatinine, phosphate, sulfate, etc., and the marked decrease in urea clearance and phenolsulfonphthalein excretion. The adrenal cortex also seems to exert a profound effect on carbohydrate metabolism, differing from the effect

[104] R. F. Loeb, D. W. Atchley, E. M. Benedict, and J. Leland, *J. Exptl. Med.*, **57**, 775 (1933).

[105] G. A. Harrop, A. Weinstein, L. J. Soffer, *ibid.*, **58**, 1 (1933).

exerted by adrenaline. Deficiency of the latter hormone, as would result from destruction of the medulla, is not associated with glycogen depletion from the liver; when adrenaline is injected and glycogen is available, the hyperglycemic response is elicited. However, when the adrenal cortex is destroyed the glycogen reserve of the liver disappears, so that the administration of adrenaline fails to raise the blood-sugar concentration.

According to Verzár [106] and associates the manifestations observed in adrenal-cortical insufficiency almost parallel those produced in iodoacetate poisoning. Many of the changes in both conditions may be explained on the basis of inhibition of phosphorylation. Verzár's views and the similar ideas of Jimenez–Diaz [107] have been favorably reviewed by Marrian and Butler.[108]

For bioassay of the active principle of the adrenal cortex Hartman employs young adrenalectomized rats. Ordinarily these either lose weight after the operation, or fail to gain; the administration of sufficient cortical extract enables them to grow and develop normally. Another test used by Hartman is based on the effect of the hormone in preventing the fall in body (colonic) temperature observed in untreated rats when exposed to cold.

According to Biskind,[109] the only satisfactory method of determining the potency of adrenal cortical preparations is by definitely demonstrating prolongation of life beyond the maximum survival period in untreated, completely adrenalectomized animals.

The dog method of assay proposed by Harrop, Pfiffner, and others [110] consists in "determining the minimum daily per kilo dose of a preparation which, when administered over a period of 7 days to adrenalectomized dogs, will maintain the blood urea nitrogen and clinical condition of the animals in an essentially normal state. The minimum daily kilo dose constitutes 1 dog unit."

Pfiffner, Wintersteiner, and Vars [111] have reported the preparation from beef adrenals by means of exhaustive fractionation of a product which assayed 400 dog units per milligram.

Chemistry of Adrenal Cortical Principles. Work is in progress in several laboratories, especially in those of Kendall, Wintersteiner, and Reichstein, which is destined to shed considerable light on the chemical constitution and physiological significance of the relatively large group of compounds that have been isolated from the adrenal cortex. A detailed account of these would be untimely as our present knowledge is likely to be considerably extended and revised in the near future.

[106] *Arch. ges. Physiol.*, **236**, 321, 693 (1935); **237**, 1, 14, 476 (1936).
[107] *Lancet*, **2**, 1135 (1936).
[108] *Ann. Rev. Biochem.*, **6**, 303 (1937).
[109] *J. Am. Med. Assoc.*, **105**, 667 (1935).
[110] *Proc. Soc. Exptl. Biol. Med.*, **29**, 449 (1932); *J. Biol. Chem.*, **104**, 701 (1934).
[111] *Ibid.*, **111**, 585 (1935).

A brief summary of some of the outstanding achievements so far is, however, appropriate.

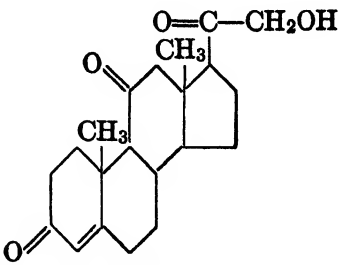
In 1935, Wintersteiner and Pfiffner [112] reported the isolation from concentrates of the cortical hormone of one nitrogenous and four non-nitrogenous compounds. The composition of these is given by the following formulas: (A) $C_{20}H_{34}O_5$, or $C_{21}H_{36}O_5$; (B) $C_{21}H_{32}O_5$; (C) $C_{24}H_{40}O_7$; (D) ?; (E) $C_{11}H_{18}O_2N_2$. One of these, compound B, was thought to be identical with the principle previously isolated by Kendall.[113]

Soon after, in a more detailed account of their work, Mason, Myers, and Kendall [114] likewise described the isolation in crystalline form of four compounds. The composition, physical properties, as well as their oxidation products were determined. A fifth compound, E, was identified by a study of its oxidation product. In a later study its isolation was reported. It appeared to be a trihydroxy diketone, $C_{21}H_{28}O_5$. The physiological activity of this compound was stated to be qualitatively, but not quantitatively, similar to that of cortin (adrenal cortex extract). Oxidation of compound E yielded a ketone, $C_{19}H_{24}O_3$, which was found to be effective in stimulating comb growth in the capon, indicating its chemical relationship to the sterols. In the meantime Reichstein [115] had isolated from the adrenal cortex a compound of the formula $C_{18}H_{24}O_3$, which proved to be about one-fifth as effective as androsterone (p. 499) in producing comb growth in capons. Accordingly he names it "adrenosterone" and suggested that its overproduction in tumors of the adrenals may be related to the development of masculinity in females so afflicted. By 1937, nine different, but closely related, compounds had been isolated by Reichstein and fellow-workers from the adrenal cortex, including corticosterone, a crystalline substance which they report possesses the biological activity of adrenal cortical extract.

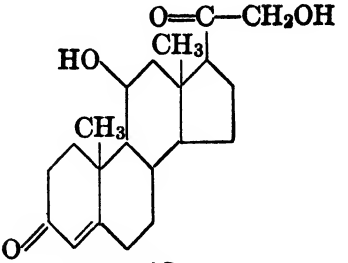
More recently the workers in Kendall's laboratory [116] added a sixth compound (H) to their list and submitted what they consider to be the probable structural formulas of components A and B. It is asserted that these have cortin-like properties and that the B compound is identical with corticosterone of Reichstein. It should be mentioned that Wintersteiner and Pfiffner [117] have also discovered two additional compounds, one, an unsaturated ketone of the formula $C_{21}H_{28}O_5$, and a monoketone of the probable composition $C_{21}H_{34}O_3$. These investigators make no claim that any of the seven compounds which they have

[112] *J. Biol. Chem.*, **111**, 599 (1935).
[113] *Proc. Staff Meetings, Mayo Clinic*, **9**, 245 (1934).
[114] *J. Biol. Chem.*, **114**, 613; **116**, 267 (1936).
[115] *Helv. Chim. Acta*, **19**, 29, 223, 401, 979, 1107 (1936); P. de Fremery, E. Laqueur, T. Reichstein, R. W. Spanhoff, and I. E. Uyldert, *Nature*, **139**, 26 (1937).
[116] *J. Biol. Chem.*, **120**, 719 (1937).
[117] *Ibid.*, **116**, 291 (1936).

isolated (up to 1936) possess any of the physiological properties associated with the adrenal cortex.



Compound A



Compound B

The occurrence of ascorbic acid (Vitamin C) in the adrenal cortex will be discussed elsewhere (p. 596).

Hyperfunction of the Adrenals. Hyperactivity, though usually associated with the existence of a tumor (hypernephroma), may also result from simple hypertrophy of the adrenal cortex. The outstanding manifestations of such hyperfunction are mainly related to sexual development. According to Hoskins,[118] the situation may be epitomized by the statement that the masculine characteristics are strongly accentuated. " In the male, the result is a paragon of virility. The female becomes a masculinized caricature of her former self."

When overactivity of the cortex arises in childhood, sexual precocity is the result. A boy of six or seven rapidly acquires the sexual development of a much older individual. There is enlargement of the testes, together with the appearance of hair in the pubic region. There may also be the beginning of the growth of a beard or mustache. Girls similarly afflicted show evidence of hypertrophy of the breasts and enlargement of the uterus; in some cases menstruation may occur. In a general way, these children may be said to resemble small men and women. In the girls thus afflicted there is usually a superimposition of masculine characteristics.[119]

Female Sex Hormones. *Estrone.* In 1923, Allen and Doisy [120] demonstrated the existence in follicular fluid of an agent capable of inducing estrus in immature female rats. Soon it became known through the work of these and other investigators that a similar substance was present in amniotic fluid, placenta, and the urine of pregnant women. From the latter, Doisy, Veler, and Thayer,[121] and Buten-

[118] R. G. Hoskins, "The Tides of Life," W. W. Norton & Co., New York. This is a readable summary of endocrinology by an authority on the subject.

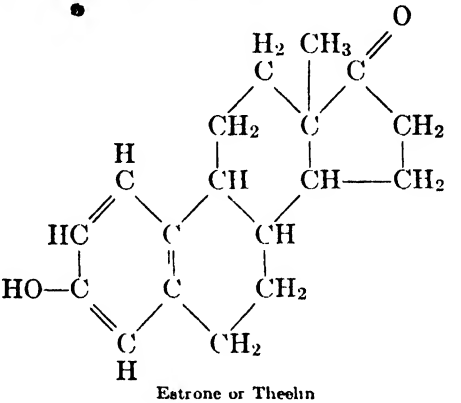
[119] The clinical aspects of hyperfunction of the adrenals have been recently summarized by W. Wolf, "Endocrinology in Modern Practice," Saunders, 1937, pp. 338, etc. See also E. J. Kepler, "Diseases of the Adrenal Glands," *Arch. Internal Med.*, **56**, 105 (1935).

[120] *J. Am. Med. Assoc.*, **81**, 819 (1923).

[121] *Am. J. Physiol.*, **90**, 329 (1929); *J. Biol. Chem.*, **86**, 499 (1930).

andt,[122] working independently, finally succeeded in isolating the hormone in crystalline form. *Theelin* (Gr. *theelus*, female) was the name originally assigned to it by Doisy, but more recently the term *estrone* has also come into general use.

Its composition is represented by the formula $C_{18}H_{22}O_2$. As shown by its structural formula, estrone is an unsaturated compound, belonging to the group of substances now designated as steroids, and containing one hydroxyl and one carbonyl group. The preparation of estrone from ergosterol has been announced.[123]



Estrone or Theelin

In 1927, Aschheim and Zondek [124] discovered that the urine of pregnant women contains large amounts of estrogenic material; indeed such urine, as well as amniotic fluid of animals slaughtered for food, were at first the main commercial sources for the hormone. It has been estimated that the average estrogenic activity of the urine of pregnant women is about 10,000 mouse units per liter, which, calculated in terms of the pure hormone (estrone or theelin), corresponds to 1 mg. Zondek [125] found in 1930 that the concentration of estrogenic substance in the urine of pregnant mares was about ten times as much as in human pregnancy urine. Even more astounding was his later discovery that the urine of stallions was an even richer source of the hormone. According to Zondek's data,[126] the sexually mature woman has a daily urinary output of estrogenic material equivalent to 170 mouse units; in the pregnant woman, the average output is 15,000 units; in the nonpregnant mare, 2,000 units; in the pregnant mare, 1,000,000 units; and in the stallion it is 1,700,000 units. From the data obtained so far it seems that only in equines (horse, zebra, ass, kiang) is this paradox of

[122] *Naturwissenschaften*, **17**, 879 (1929).

[123] R. E. Marker, O. Kamm, T. S. Oakwood, and J. F. Laucius, *J. Am. Chem. Soc.*, **58**, 1503 (1936).

[124] *Klin. Wochschr.*, **6**, 1322 (1927).

[125] *Ibid.*, **9**, 2285 (1930).

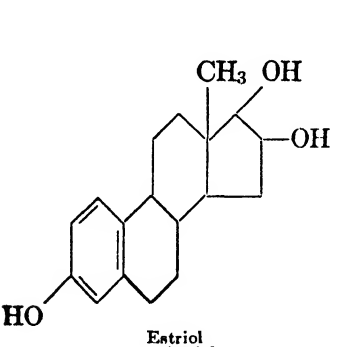
[126] *Nature*, **133**, 209, 494 (1934).

a higher concentration of female sex hormone in the male than in the female observed. The testes of stallions contain more of this hormone than the ovaries of mares.

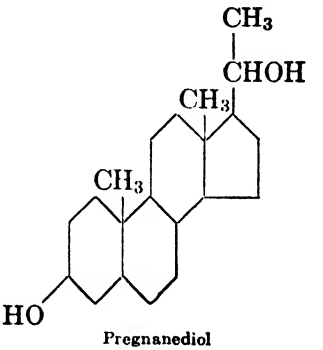
Estrone, in pure form, has been isolated from palm kernel extract (Butenandt and Jacobi [127]), and its occurrence elsewhere in the vegetable kingdom has been pointed out. It is not improbable that this and related hormones may be of significance in plant biology, but this remains to be fully established.[128]

The estrogenic activity of estrone-containing preparations or fluids, as well as of other compounds possessing this property, may be expressed in terms of the international unit. This is the quantum of activity of 0.1γ (1γ = 1 microgram = 0.001 mg.) of the standard preparation of estrone kept at the National Institute for Medical Research, London, under the auspices of the Health Organization of the League of Nations. The mouse unit,[129] widely used as a measure of activity, corresponds roughly to 1 international unit, although results reported from different laboratories vary from 0.04γ to 0.1γ of pure estrone.

Estriol and Pregnanediol. Pregnancy urine contains at least three substances chemically related to estrone. One of these is estriol (also known as theelol), the hydrate of estrone, first isolated by Marrian [130]



Estriol
or theelol



Pregnanediol

[127] *Z. physiol. Chem.*, **218**, 104 (1933).

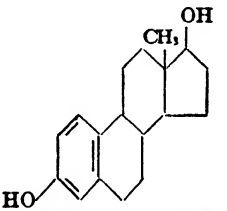
[128] For a brief account of this interesting subject, the reader is referred to L. F. Fieser's monograph, "The Chemistry of Natural Products Related to Phenanthrene," New York, 1936, Chapter V.

[129] A mouse unit of hormone is defined as the quantity which just suffices to produce estrus in the ovariectomized mouse. The rat unit is usually defined as the highest dilution of active material which, when given in three divided doses at four-hour intervals, produces estrus in the castrated mature rat at the end of three days after the first injection. Allen and Doisy's definition of the rat unit was "the minimum amount necessary to induce estrus with complete cornification of the vaginal mucosa as judged from a smear, in 75 per cent of a large group of ovariectomized, sexually mature rats. The rat unit thus defined corresponds to about 3 international units (0.3γ).

[130] *Biochem. J.*, **24**, 435, 1021 (1930).

and independently by Doisy.[131] It has the formula $C_{18}H_{24}O_3$. Unlike estrone, with which it is so closely associated, estriol is physiologically practically inert. The estrogenic effect of estriol has been estimated to be less than one-hundredth that of estrone. Marrian [132] later isolated pregnanediol, $C_{21}H_{36}O_2$, and Hartmann and Locher [133] discovered its stereoisomer, allopregnanediol. Neither of these exhibits any physiological activity.

Estradiol or Dihydrotheelin. On reducing the carbonyl group of estrone, the dihydro derivative, *estradiol*, is obtained. Like certain other compounds formed from estrone, estradiol is more potent than its mother substance. The preparation of estradiol by Schwenk and Hildebrandt,[134] as well as certain observations on the relative potency of theelin preparations made in their laboratory, led Doisy and co-workers [135] to attempt the isolation of the more active substance. From 1/2 ton of sows' ovaries a minute amount was isolated as the monobromobenzoate, and its identity to estradiol, derived chemically, was determined. Subsequently 400 liters of liquor folliculi, representing 4 tons of ovaries were processed and a somewhat larger amount of the hormone was isolated as the di-α-napthoate. It has been estimated that 1 ton of hogs' ovaries contains about 6 mg. of the more potent hormone. Estradiol is 4 to 8 times as active, physiologically, as estrone. Estradiol has also been isolated from the urine of pregnant mares.[136] The significance of this naturally occurring estrogenic compound and its biological relation to estrone remain to be more fully elucidated.



Estradiol or dihydrotheelin

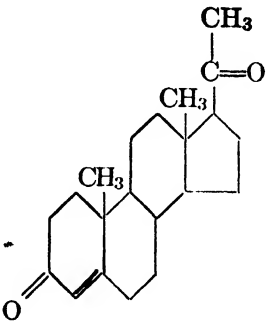
In addition to the hormones that have been described, the urine of pregnant mares contains the following: *equilin*, $C_{18}H_{20}O_2$; its isomer, *hippulin*; and *equilinin*, $C_{18}H_{18}O_2$. All three have estrogenic activity. In passing it may be mentioned that a number of synthetic compounds, derivatives of phenanthrene, exhibit this property.

[131] *J. Biol. Chem.*, **91**, 641, 655 (1931).
[132] *Biochem. J.*, **23**, 1090 (1929).
[133] *Helv. Chim. Acta*, **18**, 160 (1935).
[134] *Naturwissenschaften*, **21**, 177 (1933).
[135] D. W. MacCorquodale, S. A. Thayer, and E. A. Doisy, *Proc. Soc. Exptl. Biol. Med.*, **32**, 1182 (1935); *J. Biol. Chem.*, **115**, 435 (1936).
[136] O. Wintersteiner, E. Schwenk, and B. Whitman, *Proc. Soc. Exptl. Biol. Med.*, **32**, 1087 (1935).

The relation of estrogenic compounds to carcinogenesis has been stressed by a number of writers.

It is impossible to consider here the therapeutic uses of the estrogenic hormones.

Corpus Luteum Hormone. The hormones that have just been described are formed in the ovary, or perhaps in the ripening follicles, and those that are physiologically active are concerned in producing the characteristic changes in the uterus and vagina associated with estrus. The proliferation of the uterine mucosa induced by the follicular hormones is carried further by the hormone of the corpus luteum. The corpus luteum is a yellowish material which gradually fills the ruptured follicle after expulsion of the ovum. Proliferation of the uterine mucosa enables the reception and attachment of the ovum, if fertilized. Should this occur, the corpus luteum continues to grow; its presence is in fact essential for the successful continuation of pregnancy. It is due to the persistent corpus luteum that ovulation is suppressed. In part it may also participate in stimulating the development of the mammary glands—a view that has been challenged, however. That the various functions of the corpus luteum are due to a hormone has been long suspected, but it was not until 1934 that proof of this was obtained. In that year reports appeared in rapid succession from four widely scattered laboratories announcing the isolation of three different compounds from the corpus luteum.[137] Collectively these have been called *progestin*, but the principal hormone has been designated *progesterone*, $C_{21}(H_{30}O_2$. The other components, α- and β-progesterone, are different crystal habits of one substance. Progesterone has been prepared from two other natural products, stigmasterol (p. 81) and pregnanediol. The chemical constitution of progesterone is represented by the formula:



Progesterone

Male Sex Hormones. The profound influence of the testes on the primary and secondary sex characteristics has been known, at least

[137] A. Butenandt, *Wien. klin. Wochschr.*, **47**, 934 (1934); Slotta, Ruschig, and Fels, *Z. physiol. Chem.*, **228**, 207 (1934); *Ber.*, **67**, 1270 (1934); W. M. Allen and O. Wintersteiner, *Science*, **80**, 190 (1934); Hartmann and Wettstein, *Helv. Chim. Acta*, **17**, 878 (1934).

superficially, since ancient times, and in a sense, the hormonal mechanism of testicular function has been suspected for many years. However, the real progress of our knowledge of the subject has a much more recent beginning. Among the first important contributions was the preparation in Koch's laboratory [138] of potent extracts of the lipid fraction of bull testicles. It was shown that the injection of small amounts of the extract into capons produced striking effects on the secondary sex characters, most conspicuous of which was stimulation of comb growth. So definite was this effect that Gallagher and Koch [139] made it the basis of a method for the bioassay of testicular hormone preparations. Although there are other useful criteria of evaluating the potency of such extracts, this procedure proved of immeasurable value in many of the investigations since that time. The unit of measure is the " capon unit," which according to the definition of Gallagher and Koch " is the amount which, injected per day for 5 days, yields an average of 5 mm. increase in length and height of the combs on at least five brown Leghorn capons." [140]

Among other methods by means of which the activity of male hormone may be demonstrated are those based on the effects on spermatozoön activity and the cytological and gross changes of the seminal vesicle, prostate, Cowper's gland, and vas deferens. While such tests of potency are usually conducted on the rat, the effects are related to the normal functions of the testicular hormone (or hormones) in controlling the development of the male genital organs, the secretory activity of the accessory glands, and the development of the secondary sexual characters.

The developments in the last few years have been so rapid and so startling that an enormous literature has accumulated, which it is impossible to review here. All that will be attempted will be a brief description of three compounds that have been defined as the male sex hormones.[141]

[138] L. C. McGee, M. Juhn, and L. V. Domm, *Am. J. Physiol.*, **87**, 406 (1928); C. R. Moore and McGee, *ibid.*, **87**, 436 (1928); T. F. Gallagher and F. C. Koch, *J. Biol. Chem.*, **84**, 495 (1929); Koch, *J. Am. Med. Assoc.*, **96**, 937 (1931).

[139] *J. Pharm. Expl. Therap.*, **40**, 327 (1930); **55**, 97 (1935).

[140] A somewhat different standard has been employed by Butenandt and Tscherning,[142] who define the capon unit as the amount of substance which, when administered to each of three capons on two successive days, produces in the course of the third and fourth day an average increase of 20 per cent in the area of the comb.

In Laqueur's laboratory the comb-growth unit has been defined as the minimum daily dose which, introduced in two injections per day for four days, causes on the fifth day an increase of 15 per cent in the area of the comb in over 50 per cent of the capons. The areas may be measured from shadowgraphs, or photographs.

Other investigators have employed still different definitions, but the underlying principle of practically all procedures is essentially that introduced by Gallagher and Koch.

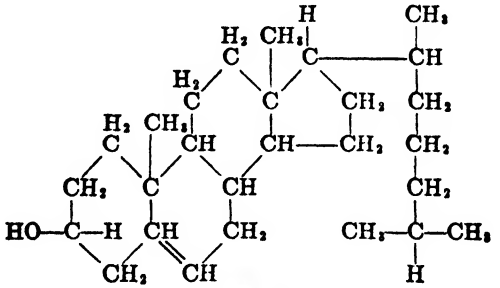
[141] The subject has been ably and comprehensively reviewed by F. C. Koch, *Physiol. Rev.*, **17**, 153 (1937).

Androsterone. The first of the male hormones was isolated from the urine by Butenandt and Tscherning,[142] 15 mg. of the crystalline hormone being obtained from 15,000 liters of urine. In later trials considerably larger yields were obtained, and it has been estimated that 1 liter of urine contains on an average approximately 1 mg. of androsterone. The actual yield of the pure hormone is, however, only a fraction of this amount.

It was determined that 150 to 200γ (micrograms) of the pure hormone is equivalent to 1 capon unit. The chemical constitution was found to be $C_{19}H_{30}O_2$, and, although the amount of material available for study was exceedingly small (about 25 mg.), Butenandt obtained sufficient information to lead him to propose the structural formula indicated below. This proved to be correct, for in 1934 Ruzicka[143] succeeded in converting cholesterol into androsterone, thereby establishing the formula.

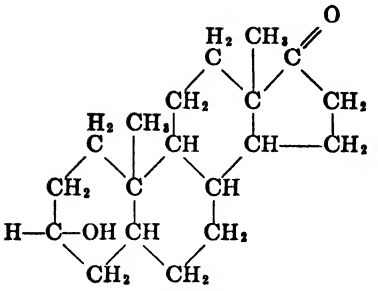
Dehydroisoandrosterone. Associated with androsterone in the urine and present in about the same amount is a second hormone, dehydroisoandrosterone ($C_{19}H_{28}O_2$), which was isolated in crystalline form (as the ester-chlor derivative) by Butenandt and Dannenbaum[144] in 1934. Soon after, its production from cholesterol was reported from two laboratories (Ruzicka and Wettstein[145]; Wallis and Fernholz[146]); and other investigators prepared it from sitosterol (Oppenhauer[147]) and stigmasterol (Butenandt, *et al.*[148]).

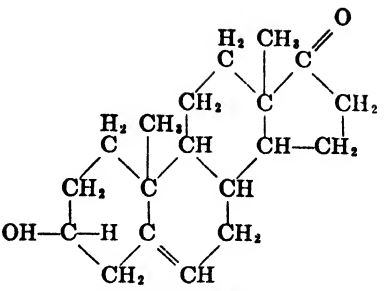
Judging by the comb-growth test, dehydroisoandrosterone is physiologically about one-fourth to one-third (one-third to one-half according to Koch) as active as anhydrosterone. The following structural formulas of androsterone and dehydroisoandrosterone indicate their chemical relationship to cholesterol.



Cholesterol

[142] *Z. angew. Chem.*, **44**, 905 (1931).
[143] *Helv. Chim. Acta*, **17**, 1389, 1395, 1407 (1934).
[144] *Z. physiol. Chem.*, **229**, 192 (1934); See also Butenandt, *et al.*, *ibid.*, **237**, 57 (1935).
[145] *Helv. Chim. Acta*, **18**, 986 (1935).
[146] *J. Am. Chem. Soc.*, **57**, 1379, 1504 (1935).
[147] *Nature*, **135**, 1039 (1935).
[148] *Z. physiol. Chem.*, **237**, 57 (1935).


Androsterone


Dehydroisoandrosterone

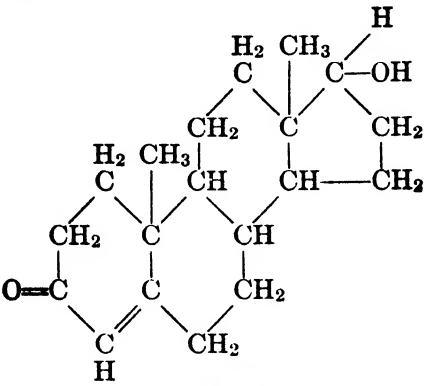
It may be noted that in regard to the degree of unsaturation dehydroisoandrosterone is intermediate between androsterone and estrone, and it has therefore been intimated that this hormone may be a natural precursor of substances in both groups (Fieser). It is also worthy of comment that the presence of the male hormone has been detected in the urine of pregnant and non-pregnant women.

Testosterone. Although the two male hormones found in the urine exhibited, at least qualitatively, the physiological properties ascribed to testicular extract, it was soon realized that they differed on a quantitative basis. Even in some of the earlier work in Koch's laboratory, it was noted that the hormone concentrate derived from bulls' testes was about ten times as active as the corresponding concentrate obtained from urine. Other observations elsewhere increased the likelihood that the hormone derived from testes and urine differed in biological activity. In Laqueur's laboratory,[149] for example, it was found that if amounts of hormone from the two sources, equivalent in respect to capon units, were injected into castrated male rats, the effect produced on the seminal vesicles (increase in size) were widely divergent (ratio of 5 : 1 in favor of the testicular extract). From this it seemed probable that the characteristic hormone of the testis was not identical with either of the two known male hormones that had been isolated from the urine. David and others [150] in Laqueur's laboratory pursued this problem and in a very short time reported the isolation of the testicular hormone, which has been designated testosterone. From 100 kg. of testis tissue was obtained 10 mg. of the pure hormone. Analysis showed it to have the composition $C_{19}H_{28}O_2$. Judging by the capon test, testosterone is 6 to 10 times as active as androsterone. Tests on castrated rats indicate that it is 25 to 30 times more effective than androsterone in stimulating the seminal vesicles and 6 times as potent in its action on the prostate.

The fact that an unsaturated diketone that had been previously derived from dehydroisoandrosterone (androstenedione, 3–17) behaved very similarly to testosterone furnished the clue to the probable mol-

[149] *Nature*, **135**, 184 (1935).
[150] *Z. physiol. Chem.*, **233**, 281 (1935).

ecular configuration of the hormone. As a result, the artificial production of testosterone from dehydroisoandrosterol was accomplished independently by Butenandt [151] and Ruzicka [152] within three months after its isolation.



Testosterone

The evidence at present indicates that testosterone is the characteristic hormone of the testis. As to the significance of the hormones found in the urine (male and female), the view has been adopted by certain writers that they are not the original hormones of testes and ovaries. Fieser [153] states, "Androsterone and estrone appear to be either transformation products of the true hormones of the genital glands or else companion substances."

Recalling how vague the subject was only a few years ago, it would seem, and justifiably so, that recent achievements have been both remarkable and enlightening and that present knowledge is explicit and fairly comprehensive. Yet in all probability more information, of a type that perhaps cannot now be even anticipated, is destined to emerge from the laboratories of those investigators who are still engaged in the problem and who by recent performance have exhibited ingenuity, acumen, and intensity of effort almost unparalleled in contemporary scientific endeavor.

The Hypophysis or Pituitary Gland; Hormones of the Posterior Lobe. The hormones of the pituitary gland constitute an extraordinarily complex problem. The multiplicity and divergence of the functions exhibited by the posterior and anterior lobes suggest the existence of a number of active principles, some of which have been separated from others; however, none has been isolated in a form suitable for identification as a chemical individual. With the progress of knowledge it has become increasingly evident that the hypophysis commands a

[151] *Ibid.*, **237**, 89 (1935).
[152] *J. Am. Chem. Soc.*, **57**, 2011 (1935); *Helv. Chim. Acta*, **18**, 1264 (1935).
[153] "Chemistry of Natural Products Related to Phenanthrene," p. 240.

dominant position because of its control over other organs of internal secretion. Lack of space makes it impossible to do more than briefly summarize the essential functions of this gland and the hormones that are believed to be involved.

The Posterior Lobe. Extracts of the posterior lobe stimulate the contraction of smooth muscle (bladder, intestine, mammary gland, uterus, etc.). Particularly striking are the contractions of the uterus (*oxytocic* effect), a property that has found important clinical applications. The oxytocic principle has been chemically separated by Kamm and associates [154] and designated by them as *α-hypophamine.* It is also referred to as *oxytocin* and *pitocin.* The effect of this hormone fraction on smooth muscle in general requires further study.

Another important effect of posterior pituitary extracts (*pituitrin*) is the elevation of blood pressure. It is not altogether clear to what extent this "pressor" effect is due to stimulation of the heart muscle and particularly of the smooth muscle of the walls of the blood vessels. However, Kamm and co-workers have separated from the posterior pituitary a hormone fraction which is devoid of the oxytocic effect and which specifically raises the blood pressure. This hormone has been designated *β-hypophamine. Vasopressin* and *pitressin* are other terms commonly used in describing it. At present, our chemical knowledge of these substances is comparatively vague.

The disease *diabetes insipidus* was formerly associated with injury to the posterior lobe. In this condition large quantities of urine are excreted; indeed a daily elimination exceeding 50 liters has been occasionally reported. The same effect on water metabolism seems to result, however, from injury to the hypothalamic region of the brain. The important consideration here is that extracts of the posterior pituitary markedly decrease the urine volume, both in these individuals and normally. An opposite effect is produced in the anesthetized animal. It is supposed that the diuretic-antidiuretic action of the posterior pituitary may be attributed to the "pressor substance." Others consider the control of the water metabolism to be dependent on a specific hormone.

Recent work has brought to light a possible interrelationship to carbohydrate metabolism. Injections of pituitrin have been shown to lessen the effect of administered insulin. This antagonistic action has been brought out even more clearly by the administration of insulin to animals deprived of the posterior lobe. Such animals exhibit a marked sensitivity to the action of insulin. There is also a definite indication of a relation of the posterior lobe to fat metabolism. Deficiency of pituitary secretion is associated with a high fat content in the blood and the deposition of fat in the tissues, leading to obesity. On the contrary, the injection of posterior lobe extracts results in a diminution in the blood fat and although there is a corresponding increase of fat in the

[154] *J. Am. Chem. Soc.*, **50**, 573 (1928).

liver, the resultant effect is a greater utilization of this substance in the body.

In short, although only two hormones have been extracted in relatively pure form from the posterior lobe thus far, the physiological properties suggest the possible existence of others.

It also appears that the pigmentation of amphibia, reptiles, and other organisms is in some way dependent on the pituitary through a controlling effect on the expansion and contraction of the melanophores, or pigment cells.

The chromatophorotropic principle has been named *intermedin*, owing to its occurrence chiefly in the pars intermedia of the pituitary gland.

Hormones and Functions of the Anterior Lobe of the Pituitary. *Growth Hormone.* Extirpation of the anterior lobe or of the entire gland, in young animals, has a marked inhibitory effect upon growth. In a celebrated experiment, Aschner [155] selected two puppies from the same litter, removed the hypophysis of one and used the other as a control. The dog without the hypophysis remained stunted, whereas the control animal grew to normal size.

Similar retardation of growth, as well as atrophy of the sex organs, occurs in rats following hypophysectomy, as shown by P. E. Smith.[156] If such dwarfed rats are given injections of anterior lobe substance, growth and the development of the sex organs are resumed. Almost normal growth was obtained by Smith in a group of hypophysectomized rats in which a daily homeotransplant of anterior lobe tissue was made.[157]

As was shown by Evans and Long,[158] if normal rats are given injections of anterior lobe extract, growth occurs at a much greater rate than normal. For example, a female rat may attain a weight of 700

[155] *Arch. ges. Physiol.*, **146**, 1 (1912).

[156] *Anat. Rec.*, **33**, 289 (1926); *Am. J. Physiol.*, **80**, 114; **81**, 20 (1927); *J. Am. Med. Assoc.*, **88**, 158 (1927). See also G. L. Foster and P. E. Smith, *J. Am. Med. Assoc.*, **87**, 2151 (1926); Smith and E. T. Engle, *Am. J. Anat.*, **40**, 159 (1927–28).

[157] A series of reviews under the title "Glandular Physiology and Therapy" have been published in the *Journal of the American Medical Association*, in which the literature on the hypophysis until 1935 has been comprehensively and critically summarized by authorities on the subject. The page references, Vol. **104** (1935), follow: H. M. Evans, "Clinical Manifestations of Dysfunction of the Anterior Pituitary," 464; "The Growth Hormone of the Anterior Pituitary," 1232; P. E. Smith, "General Physiology of the Anterior Pituitary," 548; "The Hypophyseal Gonadotropic Hormones," 553; J. B. Collip, "Interrelationships among Urinary, Pituitary and Placental Gonodotropic Factors," 556; "Diabetogenic, Thyrotropic, Adrenotropic and Parathyrotropic Factors of the Pituitary," 827, 916; O. Riddle, "The Lactogenic Factor of the Pituitary," 636; S. Aschheim, "Pregnancy Tests," 1325; R. T. Frank, "Sex-Endocrine Factors in Blood and Urine in Health and Disease," 1991. These and other articles on the subject of endocrinology have been compiled into a volume bearing the same title given above and published in 1935 under the auspices of the American Medical Association.

[158] *Anat. Rec.*, **21**, 62 (1921).

grams during the time that it takes its litter-mate sister to grow to a weight of only 300 grams. The difference in weight between the "giant" and the normal rat is due, not to a greater accumulation of fat, but to symmetrical growth of the body as a whole, including the osseous system.

These manifestations have their counterpart in man, the most conspicuous effect of hypopituitarism in early life being inhibition of growth. Human dwarfism and infantilism due to pituitary deficiency is a more or less well-recognized clinical entity. Aside from failure to grow there are other manifestations, such as sexual immaturity, owing to the lack, not only of the growth-promoting principle, but also of the gonadotropic and other hormones elaborated by the anterior lobe. The pituitary dwarf usually has a normal intellect, which distinguishes him from the cretin. In passing it may be mentioned that certain writers hold the view that cretinism itself may be the result of the depression of pituitary function. Cases of human dwarfism have been reported in which growth at a normal rate was induced by treatment with pituitary extract.

Hyperactivity of the anterior lobe of the hypophysis usually results from the development of an adenoma of the gland. The clinical manifestations of such overactivity are well defined. If the condition develops before union of the epiphyses has taken place, that is in very young individuals, there is in consequence growth of the long bones to gigantic proportions, and *gigantism* is the result. Most giants of the circus are victims of this disease. The height usually attained is between 6.5 and 8 feet, but there are less authentic records of much taller individuals (9 and 9.2 feet).

After epiphyseal union has taken place, symmetrical growth is no longer possible. In consequence the skeletal changes consist in an overgrowth of certain parts only. The jaw enlarges and protrudes, the spine is bowed, and the hands and feet increase in size. Because of the associated connective tissue hyperplasia, the nose is widened, the lips, tongue, scalp, and skin generally become greatly thickened. The altered appearance has been likened to a "reversion to the gorilla type." Such in part is the picture of *acromegaly*. It generally begins in early middle life. In an individual of 20 years of age, or older, hyperpituitarism of the severity described would give the typical picture of acromegaly. If the condition should develop in early adolescence, acromegalic characteristics are superimposed upon those of gigantism.

Inasmuch as the anterior part of the gland contains the gonad-stimulating hormone, hyperfunction as in gigantism and acromegaly should be associated with increased sexual virility, and in fact this may occur at first. But as the disease runs its course, sexual activity diminishes or disappears, for not infrequently the condition of hyperfunction gives way to hypofunction. However, the changes in the skeleton being fixed, it is obvious that they cannot be undone.

Acromegaly has been produced experimentally in dogs as a result of long-continued administration of the growth-promoting fraction of anterior lobe extract.

The growth-promoting hormone has not been isolated in pure form and there is therefore no certain knowledge of its chemical identity. However, very active and relatively pure preparations have been obtained by Van Dyke,[159] Collip [160] and others. In Van Dyke's procedure the hormone is extracted with dilute alkali; extraneous material is precipitated by adjusting the *p*H to 7.2, and the active principle which remains in solution is salted out with sodium sulfate. After further purification, the active fraction is taken up in water, in which form it is administered. The potency of the product may be assayed by its growth-promoting effect on hypophysectomized rats.

From its properties the growth hormone is either a protein, or so closely associated with protein that their separation has not been possible. To designate it, Van Dyke and Wallen-Lawrence have proposed the name *phyone* (φύω, I cause to grow).

Thyrotropic Hormone. A host of investigators have described the interrelationships between the hypophysis and thyroid. It has been found that hypophysectomy causes thyroid atrophy, a reduction of basal metabolism, and other evidences of thyroid deficiency, whereas the administration of extract of the anterior lobe produces thyroid hyperplasia and increased metabolism. Indeed, practically all the manifestations of hyperthyroidism, including exophthalmos (in the guinea pig), depletion of liver glycogen, reduction of the iodine content of the thyroid, and increased urinary output of creatine and calcium have been observed. As these effects are not obtained in animals deprived of their thyroid, it has been concluded that the anterior lobe contains a hormone capable of stimulating the thyroid. A number of writers have even considered the possibility that hyperfunction of the pituitary may be the primary factor in the hyperthyroidism of Graves' disease.

Extracts of the anterior lobe have been fractionated with practically complete separation of the thyrotropic and growth-promoting factors.

Parathyrotropic Hormone. Proliferation of the parathyroid cells occurs as a result of the administration of anterior lobe extracts, according to a number of investigators, and it has also been observed, though less consistently, that the parathyroid undergoes atrophy in hypophysectomized animals. Even more conflicting is the information concerning the influence of pituitary extract in raising the serum calcium, but those who have observed it in the normal animal by using fractions presumably rich in respect to the parathyrotropic principle could not obtain this response in the parathyroidectomized animal.

[159] *J. Pharm. Exptl. Therap.*, **40**, 413 (1930); **43**, 93 (1931).

[160] J. B. Collip, H. Selye, and D. L. Thomson, *Proc. Soc. Exptl. Biol. Med.*, **30**, 544 (1933).

The literature on the parathyrotropic hormone has been summarized by Collip.[157] Houssay [161] has submitted evidence for his view that the functions of the parathyroid, hypophysis, and pancreas are closely interrelated.

Pancreatropic Hormone. The relevant information at hand is that the pancreas undergoes atrophic changes in hypophysectomized dogs, whereas the contrary effect, namely proliferative changes of the islet tissue, are produced as a result of the administration of anterior pituitary extract. Stimulation of the pancreas by means of such extracts tends to lower the blood sugar. A further indication of stimulation is glycogen depletion from the liver.

Diabetogenic Hormone. Aside from the pancreatropic principle, the hypophysis is believed to contain an agent, the "diabetogenic hormone," which is capable of raising the level of blood sugar, apparently by releasing glucose from liver glycogen. Extirpation of the hypophysis tends to lower the blood sugar; this effect is especially striking in the pancreatectomized animal, as exemplified in the Houssay dog (hypophysectomized-depancreatized). Removal of the hypophisis of a depancreatized, diabetic animal results in at least temporary amelioration of the symptoms of diabetes. Injection of anterior pituitary extract containing the diabetogenic hormone produces a marked rise in the blood sugar of Houssay dogs.

Certain writers are inclined to associate the effects of the diabetogenic hormone with disturbance in fat metabolism, and particularly with the production of acetone bodies, since fairly pure extracts of the diabetogenic substance not only raise the level of blood sugar in Houssay dogs but also produce an acetonuria in fasted, or in fat-fed normal animals (Collip). However, partial separation of the two principles seems to have been accomplished, the diabetogenic substance being removed in a fraction containing the growth hormone, while the ketogenic substance appears in the same fraction with the thyrotropic hormone. On indirect evidence, Collip has brought out the fact that the last two, the ketogenic and thyrotropic principles, are not identical.

Adrenotropic or Interrenotropic Hormone. The existence of an adrenal-stimulating principle in the hypophysis is based on essentially the same kind of evidence invoked in support of the other hormones that have been described. The essential information seems to be: atrophy of the adrenal cortex following hypophysectomy, which may be counteracted by the injection of anterior pituitary extract; and cortical hypertrophy which usually results from an overabundant supply of the extract.

Clinical experience is in agreement with experimental findings, since it has been found that destruction of the pituitary is associated with adrenal atrophy. Various authors have commented on the possibility that certain cases of Addison's disease may be secondary to pituitary

[161] *Harvey Lectures*, 31, 116 (1935–36).

deficiency, and indeed some have noted improvement after treatment with adrenotropic extract. The frequent occurrence of cortical hypertrophy in acromegaly is also of significance in this connection.

Lactogenic Hormone, or Prolactin. From the residue discarded in the separation of the growth hormone, Riddle [157] has obtained a fraction which is devoid of gonad-stimulating property, but which exerts a marked effect in promoting the enlargement and function of the mammary gland. During pregnancy, the release of prolactin is believed to be inhibited by the presence in the blood of high concentrations of ovarian and placental hormones. With the fall in concentration of these substances following delivery, the secretion of prolactin by the pituitary is presumably augmented. It also seems that prolactin contributes to the maternal behavior of rats and this may be induced even in virgin animals. The injection of the hormone into hens increases their predisposition to broodiness.

Of apparent significance is the report by Young [162] that the hyperglycemic (diabetogenic) principle of the anterior pituitary is concentrated in the prolactin fraction. Owing to the carbohydrate utilization in the production of milk the existence of a sensitive mechanism capable of promoting glycogenolysis may be a factor of considerable importance in the maintenance of an adequate supply of carbohydrate to the mammary gland without causing hypoglycemia.

Gonadotropic Hormones. One of the hormones elaborated by the anterior pituitary promotes follicle maturation in the female and proliferation of the seminal vesicle epithelium in the male. A second gonadotropic hormone promotes corpus luteum formation, but this effect is dependent on the preliminary action of the follicular hormone. When the follicles have been stimulated, the luteinizing hormone produces its characteristic effect. Separation of the follicle-stimulating from the luteinizing principle has been achieved by a number of investigators.

The importance of these hormonal factors is brought out by the fact that hypophysectomy causes atrophy of the reproductive system. However, even after these changes have begun, restoration of gonadal function may be obtained by hypophyseal implants, or by the administration of pituitary extract. Moreover, sexual precocity may be induced in immature animals by the administration of anterior lobe extract. It is believed that the gonadotropic hormones of the pituitary act primarily on the ovary and testes and that through these organs the rest of the reproductive apparatus is stimulated.

Anterior-Pituitary-Like Hormone. Aschheim and Zondek discovered that the urine of pregnant women contains a hormone, which when injected into immature mice produces (1) follicular maturation; (2) hemorrhage into the follicles; (3) formation of corpora lutea, with retention of the ova. Similar changes may be produced with human pregnancy

[162] *J. Physiol.*, 87, 13P (1936).

urine in the ovaries of rabbits, the first two reactions constituting a test of pregnancy.

It was originally supposed that the gonadotropic principle of pregnancy urine, which also occurs in the blood of pregnant women, as well as in the placenta, was formed in the anterior lobe of the pituitary. This view has been abandoned, since it has been found that the urinary hormone cannot replace pituitary extract in hypophysectomized rats. The urinary hormone, though it exerts an effect on the interstitial cells of ovary and testis, has little, if any, effect on the germinal epithelium. Evans, in his review of the subject, has summarized the evidence in support of the contention that the gonadotropic factor of the urine and the blood in pregnancy is a product of the chorionic tissues of the placenta. From this standpoint it is of significance that the pregnancy hormone occurs in the urine in cases of hydatidiform mole and chorionepithelioma. In males, its occurrence in the urine has been associated with certain embryonal (teratoid) tumors of the testis.

The present view is that the output of gonadotropic hormones by the anterior pituitary is actually diminshed during pregnancy.

In addition to the gonadotropic hormones of the anterior lobe and the pituitary-like hormones found in the urine and blood during pregnancy, there is found in the urine of ovariectomized women and of those who have normally reached the menopause an active principle which has been designated "prolan A." It stimulates growth of the Graafian follicle, without transforming it into a corpus luteum. This hormone is thought to be identical with the follicle-maturation hormone of the anterior lobe.

From the foregoing brief and incomplete discussion the important fact emerges that, in addition to its somatotropic (growth-promoting) rôle, the anterior lobe is closely associated in the functions of the thyroid, parathyroid, pancreas, adrenals, gonads, mammary gland, and either directly or indirectly with other organs, such as the liver. The dominance of the pituitary is all the more remarkable if the size of the gland is considered. In man, the anterior lobe weighs only about 0. 5 gram.

Unfortunately very little is known at present of the chemistry of the hormones of this gland. Some progress has been made, however, in the fractionation of anterior lobe extracts and in the separation of certain of the active principles. Further work may possibly reveal more of the chemistry of these hormones and may also clarify other aspects of the subject which are at present obscure or unsettled.

Antihormones. Collip has advanced the doctrine that under normal conditions the action of hormones in the organism is held in check or in balance by the presence of antihormones. Oversecretion, or an excessive supply of hormone from the outside, presumably stimulates extra production of antihormone. This is exemplified by the refractoriness which rats and other animals develop after continued administration of the growth-promoting, thyrotropic, or gonadotropic fractions of anterior

pituitary extracts. The blood serum of such refractory animals, when injected into normal, untreated rats is said to increase markedly their tolerance for the corresponding hormone. Experimental evidence confirming Collip's theory has been submitted by a number of investigators. However, others have put forward objections to this theory. Werner,[163] for example, found that refractoriness induced to thyrotropic hormone fractionated by one method does not affect the tolerance to the same hormone obtained by another method. It has also been reported [164] that refractoriness to a certain hormone preparation (anterior pituitary-like hormone of pregnancy urine) may be induced, irrespective of whether it is active or inactive. These observations suggest that the mechanism may be one of immunity; in short, that the so-called antihormones are in reality antigens formed in response to the administration of foreign protein. Collip considered this possibility, but dismissed it. In view of the conflicting evidence and the unsettled state of the subject, no definite conclusions can be drawn at this time.

Thymus. Although there are numerous reports associating the thymus with growth, the evidence on the whole does not seem conclusive that its function is hormonal in nature. It is known, however, that the gland is proportionately larger at birth than at any other time, that it continues to grow at a diminishing rate until puberty, after which it undergoes involution. This has led to the assumption that the function of the thymus may be to exert a controlling effect on sexual development, holding it in check during early life. It is of interest that thymic involution is delayed as a result of castration.

The influence on growth is illustrated by Gudernatsch's [165] early experiments in which it was observed that feeding thymus stimulated the growth of tadpoles, at the same time delaying metamorphosis. In more recent years, Rowntree and associates [166] have called attention to the accruing acceleration in growth and development in successive generations of rats under continuous treatment with thymus (calves) extract. They found that the continued administration of thymus extract increased the fertility of the parent rats. The offspring of the treated rats grew at an accelerated rate and reached maturity in advance of control animals. If the injections were continued without interruption through succeeding generations this effect was amplified. If the injections were interrupted, however, the accrued effects were nullified. To illustrate, in treated rats of the fifth generation, the teeth erupted on the first day, the eyes opened by the third day, at which time the body was already covered with fur. The testes descended by the fourth or fifth day; the vagina opened in 20 days, and some cast litters as early as the fortieth day.

[163] *Proc. Soc. Exptl. Biol. Med.*, **34**, 390, 392 (1936).
[164] G. H. Twombley, *Endocrinology*, **20**, 311 (1936).
[165] *Am. J. Anat.*, **15**, 431 (1913–14).
[166] *J. Am. Med. Assoc.*, **103**, 1425 (1934); *ibid.*, **105**, 592 (1935).

The opposite effect, namely, accruing retardation of growth and development of the offspring, resulted from the removal of the gland from successive generations of rats.[167] Thymectomy did not seem to affect the operated animals, but exerted a profound effect on the offspring. If thymectomy was omitted in one generation, the effects that accrued in preceding generations were nullified. These extraordinary observations, which other investigators have been unable to reproduce, require further confirmation and elucidation. The suggestion has been made that the thymus may be of importance as a factor in racial development.

Pineal Gland. It is uncertain that the pineal body is an organ of internal secretion. This small gland is situated underneath the posterior end of the corpus callosum. For a more precise definition of its location, the reader is referred to textbooks on anatomy.

Involution of the gland begins at about 7 years of age and ends at puberty. From this it has been surmised that the pineal like the thymus exerts a controlling effect on sexual development. This finds support in the observation that tumors of the pineal occurring in the young are often associated with sexual precocity.

Rowntree [168] has reported on the effects of continued administration of bovine pineal extract to rats. In the parents the injections increased frequency of breeding, while in the offspring of later generations the results were: decreased birth weight, decreased rate of growth, acceleration of differentiation and development. In the fifth generation the average rat was less than 50 per cent of normal in weight, but sexual maturity was attained in about half the normal time. Eruption of the teeth, opening of the eyes, and development of the fur occurred much sooner than normal.

These observations, if confirmed, should lead to interesting speculations. The same applies to the results that have been obtained in the experiments on the influence of the thymus.

Auxins. This term refers to certain growth-promoting substances, or "growth hormones," which have recently been shown to play an important rôle in plant life. The best known of these compounds are: auxin A (auxentriolic acid), $C_{18}H_{32}O_5$; auxin B, $C_{18}H_{30}O_5$, and β-indolylacetic acid.[169]

[167] N. H. Einhorn and L. G. Rowntree, *Endocrinology*, **20**, 342 (1936); **21**, 659 (1937).

[168] L. G. Rowntree, J. H. Clark, A. Steinberg, and A. M. Hanson, *Endocrinology*, **20**, 348 (1936).

[169] For further information the reader is referred to K. V. Thimann, *Ann. Rev. Biochem.*, **4**, 545 (1935); F. W. Went and K. V. Thimann, "Phytohormones," Macmillan, New York, 1937.

CHAPTER XVII

ANIMAL CALORIMETRY

The unit of measurement of heat in animal calorimetry is the large or kilogram Calorie. It is the amount of heat required to raise the temperature of 1 kg. of water from 15° to 16° C. When 4185×10^7 ergs (work units) are changed or dissipated into heat, 1 Calorie is formed.

The calorific value of organic compounds is usually determined by means of the bomb calorimeter. The essential part of this apparatus is a combustion bomb in which is supported a platinum capsule. The latter is used as the container for the material to be analyzed. A wire having electrical connections with the outside dips into it. Before an analysis, the bomb is closed tightly, filled with oxygen under a pressure of 20 to 25 atmospheres, and placed in a vessel containing water. Passing an electric current through the wire causes it to glow, thereby igniting the material in the platinum capsule. The heat evolved is calculated from the observed change in the temperature of the water.

Calorific Value of Foodstuffs. The combustion calorimeter has been used extensively in determining the heat values of foodstuffs. Somewhat more elaborate methods are required for the determination of the calorific value of foods when burned in the body. These will be discussed presently. The combustion of 1 gram of a monosaccharide yields about 3.75 carlories.[1] One gram of a disaccharide yields 3.95 calories; and a gram of starch, 4.23 calories. Hence, 4.0 or 4.1 is usually taken as the average calorific value of 1 gram of carbohydrate. This amount of heat is evolved whether the combustion occurs in the air or inside the body. The heat value of fats is considerably higher. Approximately 9.3 calories are obtained on combustion of 1 gram of fat. Here also, the amount of heat produced is the same whether the fat is burned in a bomb calorimeter or in the body. The situation is somewhat different in the case of the proteins. When burned in the bomb calorimeter, 1 gram of protein yields on an average about 5.7 calories, but in the body, the heat of combustion is found to be only about 4.1 calories. Proteins may differ somewhat in calorific value. Thus, casein produces 4.4 calories, whereas the vegetable proteins yield about 4.0 calories. The divergence in the calorific values of protein when burned outside and inside the body is due to the fact that pro-

[1] In this chapter, unless specified otherwise, the term "calorie" will be used in referring to the large or kilogram calorie.

tein combustion in the tissues is never complete. The end-products of protein metabolism (urea, etc.), though of no value as energy producers in the body, are capable of undergoing further combustion in the bomb calorimeter. One gram of urea, for example, on oxidation, yields 2.52 calories. It has been determined that on a mixed diet the ratio of carbon to nitrogen in the urine is about 0.75, and that a gram of urinary nitrogen is equivalent to 8.09 calories. This value is not constant, being influenced by a variety of factors. Following the ingestion of large amounts of carbohydrate, the urine, though practically free from sugar, may contain sufficient amounts of intermediary products of glucose metabolism to increase the calorific equivalent of a gram of urinary nitrogen to as much as 13 calories. After meat has been eaten, the calorific value of 1 gram of urinary nitrogen is 7.46 (Rubner, cited by Lusk [2]); during starvation, it is 8.49 calories.

Heat Production and Respiratory Exchange. Total energy metabolism in the body may be determined by direct or indirect methods of calorimetry. The direct method consists in placing the individual in a suitably constructed chamber and measuring the amount of heat evolved. In principle, animal and bomb calorimeters are similar. By the indirect method, the heat given off is computed from the respiratory exchange. If it is desired to determine only the rate of energy metabolism, all that is necessary is a suitable apparatus by means of which the amount of oxygen consumed during a given interval may be accurately measured. By determining the consumption of oxygen, the elimination of carbon dioxide, and the excretion of nitrogen in the urine, the necessary data are obtained for calculating, not only total heat production, but the nature and amount of each of the substances metabolized, as well.

Various forms of apparatus have been designed for the measurement of heat production in man and animals either by the direct or indirect method. Pettenkofer and Voit constructed a respiratory chamber which was used in Germany in many of the classical experiments on man. For similar studies on the dog, Rubner devised an even more efficient respiratory chamber. In the United States Atwater and Rosa [3] invented a very accurate calorimeter for use in experimental studies on human subjects. This apparatus, improved by Benedict,[4] measures heat production and respiratory exchange simultaneously.

Principle of the Atwater-Rosa-Benedict Respiration Calorimeter. The respiration calorimeter is shown in Fig. 35, and is diagrammatically represented in Fig. 36. There are three walls, an inner and an outer copper wall and an insulating wall. The two copper walls are separated from each other by a dead-air space. A similar space separates the outer copper wall from the insulating wall. The latter is constructed

[2] G. Lusk, "Science of Nutrition," 1928 edition, p. 39.

[3] Atwater and Rosa, Report of the Storrs Agr. Exp. Sta., p. 212 (1897).

[4] Atwater and Benedict, Carnegie Inst. of Washington Pub., No. 42 (1905); Benedict and Carpenter, *ibid.*, No. 123 (1910).

of two layers of compo-board separated by a layer of cork. Between the insulating wall and the outer copper wall are water pipes, along which run resistance wires, carrying an electric current. Thus the temperature of the interspace, as well as that of the outer copper wall, may be kept under control by the passage of either warm or cold water through the pipes. It is essential that the temperature of the two copper walls be maintained the same, for otherwise there would be an exchange of heat between them and, hence, either a gain or loss of

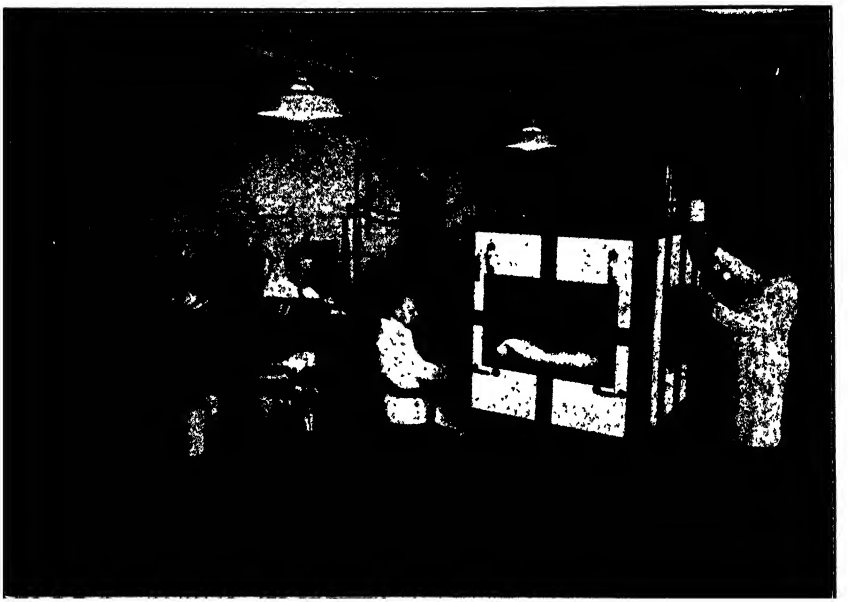


FIG. 35.—The Respiration Calorimeter of the New York Hospital (after a photograph loaned by Dr. Eugene F. Du Bois).

heat by the inner wall. Thermocouples are arranged between the two walls to determine their temperature. During the course of an experiment, temperature readings are taken at intervals of about four minutes.

Inside the calorimeter, the temperature is maintained practically constant by passing a current of cool water through a series of pipes. The heat lost by an individual in the calorimeter through radiation and conduction is thus removed by the water. The total volume of water passing through the calorimeter is measured. Likewise, the temperature of the ingoing and outgoing stream of water is recorded at short intervals during the experiment. A considerable amount of heat is used in the evaporation of water. The water evaporated from the skin and the water vapor in the expired air are taken up by sulfuric acid absorbers outside the chamber. From the amount of water thus collected, the latent heat is calculated. It is estimated that about one-

quarter of the total heat produced by the human body is present as latent heat in the water vapor which is given off. Although the temperature of the air entering the calorimeter is always heated to exactly

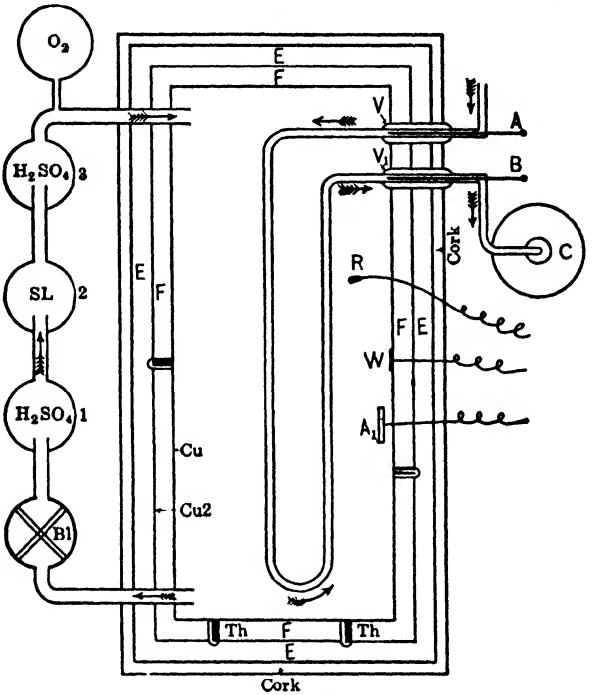


FIG. 36.—Schematic diagram of the Atwater-Rosa-Benedict respiration calorimeter. (After Graham Lusk, "Elements of the Science of Nutrition," Saunders & Co., 1928 edition, p. 70.)

Ventilating System:
- O_2, Oxygen introduced as consumed by subject
- 3, H_2SO_4 to catch moisture given off by soda lime.
- 2, Soda lime to remove CO_2.
- 1, H_2SO_4 to remove moisture given off by patient.
- Bl, Blower to keep air in circulation.

Indirect Calorimetry:
- Increase in weight of H_2SO_4 (1) = water elimination of subject.
- Increase in weight of soda lime (2) + increase in weight of H_2SO_4 (3) = CO_2 elimination.
- Decrease in weight of oxygen tank = oxygen consumption of subject.

Heat-absorbing System:
- A, Thermometer to record temperature of ingoing water.
- B, Thermometer to record temperature of outgoing water.
- V, Vacuum jacket.
- C, Tank for weighing water which has passed through calorimeter each hour.
- W, Thermometer for measuring temperature of wall.
- A_1, Thermometer for measuring temperature of the air
- R, Rectal thermometer for measuring temperature of subject.

Direct Calorimetry:
- Average difference of A and B × liters of water + (g. water eliminated × 0.586) ± (change in temperature of wall × hydrothermal equivalent of box) ± (change of temperature of body × hydrothermal equivalent of body) = total calories produced.

Th, thermocouple; Cu, inner copper wall; Cu_2, outer copper wall; E. F. dead air-spaces.

the same temperature as the air leaving it, nevertheless, the temperature of the calorimeter is determined at the beginning and end of an experiment and a correction introduced, if necessary. Another correc-

tion may be necessary, should a change in the body temperature occur. The chamber has a port-hole with inner and outer airtight doors. By opening these, one at a time, food may be passed in and excreta removed, without loss or gain of heat.

So well have the technical details of the calorimeter been worked out that when a given amount of alcohol is burned in it, and the heat production measured, the value obtained is practically identical with that found when the combustion is carried out in a bomb calorimeter. This is referred to as the alcohol check. Another way of testing the accuracy of the apparatus is to generate within it a measured amount of heat by means of an electric current. When everything is in order and functioning properly the heat produced in this way may be completely accounted for by calorimetric measurement. This is the so-called electric check.

Moreover, there is very close agreement in the results obtained by the direct and indirect methods. Atwater and Benedict [5] compared the average results per day, obtained for three individuals who were experimented upon for 40 days each, and found an average difference of only 0.2 per cent.

	Average Calories per Day
Indirect calorimetry	2717
Direct calorimetry	2723
Difference	0.2 per cent

Murlin and Lusk [6] performed a series of twenty-two experiments on a dog and found the average difference in the results obtained by direct and indirect calorimetry to be only 0.6 per cent.

In the determination of respiratory metabolism by the indirect method the equipment required is much simpler than in direct calorimetry. The accuracy of the indirect method and the value of the data especially in the study of metabolic disorders have encouraged the invention of various types of machines for clinical use. Most of these provide a graphic record of the oxygen consumed by the subject during a given interval, as well as the depth, regularity, and rate of respiration. From the value for oxygen consumption per minute and other data, the rate of metabolism may be computed.[7]

[5] Cited by Lusk, "Science of Nutrition," 4th edition, p. 62. The student is referred to this book for a detailed description of the Atwater-Rosa-Benedict calorimeter.

[6] *J. Biol. Chem.*, **22**, 15 (1915).

[7] Some of the methods employed in the measurement of respiratory exchange in man and animals are to be found in the monograph by A. Krogh, "The Respiratory Exchange of Animals and Man," Chap. II, London and New York, 1916. See also Du Bois, "Basal Metabolism in Health and Disease," Lea & Febiger, Philadelphia, 1936, and W. M. Boothby and I. Sandiford, "Laboratory Manual of the Technique of Basal Metabolism Determination," Philadelphia, 1920. The student is also referred to H. B. Richardson's review, "The Respiratory Quotient," in *Physiol. Rev.*, **9**, 61 (1929).

Respiratory Quotient, Influence of Metabolism. The ratio between the carbon-dioxide output and the oxygen intake is termed the "respiratory quotient (R. Q.)" Early in the history of the science of nutrition, it was realized that this ratio was profoundly affected by the character of the material metabolized and that, therefore, the determination of the respiratory quotient would yield information concerning the nature of the substances which were being utilized. In the combustion of carbohydrate, the volume of carbon dioxide produced is equal to the volume of oxygen used. The respiratory quotient, $\frac{CO_2}{O_2}$, is therefore 1.

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$$

The combustion of a fat (triolein) may be represented by the equation:

$$C_{57}H_{104}O_6 + 80O_2 = 57CO_2 + 52H_2O$$

Hence, the respiratory quotient of triolein is

$$\text{R. Q.} = \frac{57}{80} = 0.71$$

There are slight variations in the respiratory quotients of different fats, owing to the differences in molecular weight. For tripalmitin, the quotient is 0.703; for human fat, 0.713, etc.

It is somewhat more difficult to represent the oxidation of protein. The respiratory quotient may be computed as follows (Loewy [8]):

100 grams of meat protein contain:

52.38 grams C
7.27 grams H
22.68 grams O
16.65 grams N
1.02 grams S

Of these amounts, the following portions are excreted in the urine and feces:

Urine	Feces
9.406 grams C	1.471 grams C
2.663 grams H	0.212 gram H
14.099 grams O	0.889 gram O
16.28 grams N	0.37 gram N
1.02 grams S	

This leaves a residuum for the respiratory process of:

41.50 grams C
4.40 grams H
7.69 grams O

[8] Oppenheimer's "Handbuch der Biochemie," 4, 1, 279 (1911); cited by Lusk, 4th edition, p. 64.

The amount of oxygen here is sufficient to oxidize 0.961 gram of hydrogen, leaving for further oxidation

41.50 grams C
3.439 grams H

To oxidize these would require 138.18 grams of oxygen. The carbon-dioxide production would be 152.17 grams. These values may be converted into their volume equivalents, since 1 gram of oxygen occupies 0.699 liter and 1 gram of carbon dioxide 0.5087 liter. Computing for the value of the respiratory quotient:

$$\frac{77.39 \text{ liters } CO_2}{96.63 \text{ liters } O_2} = 0.801$$

If the combustion of carbohydrate alone were possible, the respiratory quotient would be 1; if only protein were being burned, it would be about 0.80–0.82; if fat, about 0.71. Under certain conditions, values outside the range 0.7 to 1.0 have been observed. Indeed, a quotient as high as 1.38 was obtained by Bleibtreu [9] on geese which were being stuffed and which were presumably converting carbohydrate (an oxygen-rich substance) into fat (an oxygen-poor substance). This process obviously involves the liberation of oxygen. Before hibernating, animals also show high respiratory quotients. Values lower than 0.70 have been observed in hibernating animals, the calorific requirements of which are obviously met by the stored fat. The glycerol arising in fat metabolism in these animals is probably converted, at least in part, into sugar. In diabetes, the respiratory quotient is low, since carbohydrate metabolism is deficient; and when the condition is especially severe, it may fall somewhat below 0.7 as a result of the conversion of amino acids and glycerol into glucose. In phlorhizin diabetes, Chambers and Deuel [10] observed a reduction in the respiratory quotient after the administration of glycerol, for, in the conversion of the latter into glucose, oxygen was required. Under ordinary conditions, the respiratory quotient is about 0.85, but it may vary within rather wide limits. The Hindoos and Chinese, who live largely on rice, are said to have a high respiratory quotient (above 0.9).

The amount of protein represented by 1 gram of urinary nitrogen requires for oxidation 5.923 liters (8.471 grams) of oxygen and produces 4.754 liters (9.347 grams) of carbon dioxide (Zuntz and Schumberg [11]). The calorific equivalent of 1 gram of urinary nitrogen (about 6.25 grams protein) is 26.51 calories (Lusk [12]).

Given the total oxygen consumption, carbon-dioxide production, and urinary-nitrogen elimination for a certain period, it is possible to

[9] *Arch. ges. Physiol.*, **85**, 345 (1901).
[10] *J. Biol. Chem.*, **65**, 21 (1925).
[11] "Studien zu einer Physiologie des Marsches," Berlin, 1901.
[12] For further details and other methods of calculation, consult the books by Lusk and Du Bois, previously cited.

calculate the amounts of protein, fat, and carbohydrate metabolized during that period. This may be illustrated by a simple example.

Suppose that Mr. A during a period of 24 hours consumed 400 liters of oxygen and eliminated 340 liters of carbon dioxide and 12 grams of nitrogen. For the combustion of the protein represented by the urinary nitrogen,

$12 \times 5.923 = 71.076$ liters of oxygen were used, and
$12 \times 4.754 = 57.048$ liters of carbon dioxide produced

Subtracting these values from the total volumes:

Total O_2 used	400 liters
O_2 used for protein	71 liters
O_2 used by carbohydrate and fat	329 liters
Total CO_2 produced	340 liters
CO_2 produced by protein	57 liters
CO_2 produced by carbohydrate and fat	283 liters

The ratio $\frac{283}{329} = 0.86$ is the non-protein respiratory quotient. From this figure may be computed the relative amounts of carbohydrate and fat used, or these may be determined more simply from the table on p. 518. It is to be noted that, when the non-protein R. Q. is 0.86, 1 liter of oxygen is equivalent to 0.622 gram of carbohydrate and 0.249 gram of fat. Hence, 329 liters of oxygen are equivalent to $329 \times 0.622 = 204.6$ grams of carbohydrate and $329 \times 0.249 = 81.92$ grams of fat. Accordingly, during the period of experimentation, the following amounts were utilized:

Protein (12×6.25)	75 grams
Fat	81.92 grams
Carbohydrate	204.6 grams

From these figures may be calculated the total calorific output:

$[75 \times 4.1] + [81.92 \times 9.3] + [204.6 \times 4.1] = 1908.3$ calories

According to Loewy:

1 liter of O_2 used in oxidation of protein corresponds to	4.485 calories
1 liter of O_2 used in oxidation of fat corresponds to	4.686 calories
1 liter of O_2 used in oxidation of carbohydrate corresponds to	5.047 calories

Referring to Table LIV, it will be seen that, when the non-protein respiratory quotient is 0.86, 1 liter of oxygen corresponds to 4.875 calories. From this value and the figure given by Loewy for protein, the calorific output may be computed readily as follows:

$$[71 \times 4.485] + [329 \times 4.875] = 1922.3 \text{ calories}^{18}$$

[18] The slight discrepancy between this value and 1908.3 calories (less than 1 per cent) is due to the somewhat divergent factors introduced by different authorities and to dropping, in the calculations, of the last decimal places by the present author

These calculations illustrate the method for computing heat production by indirect calorimetry.

TABLE LIV

THE SIGNIFICANCE OF THE NON-PROTEIN RESPIRATORY QUOTIENT AS REGARDS THE HEAT VALUE OF 1 LITER OF OXYGEN, AND THE RELATIVE QUANTITY IN CALORIES OF CARBOHYDRATE AND FAT CONSUMED (ZUNTZ AND SCHUMBERG, MODIFIED BY LUSK,[14] MODIFIED BY MCCLENDON[15])

One Liter of Oxygen is Equivalent to

Non-Protein Respiratory Quotient	Grams		Calories
	Carbohydrate	Fat	
0.707	0 000	0.502	4 686
0.71	0 016	0 497	4.690
0.72	0 055	0 482	4.702
0.73	0 094	0.465	4.714
0.74	0.134	0 450	4.727
0.75	0.173	0.433	4.739
0.76	0.213	0 417	4.751
0.77	0 254	0 400	4.764
0.78	0.294	0 384	4.776
0.79	0 334	0.368	4.788
0.80	0 375	0 350	4.801
0.81	0 415	0 334	4.813
0.82	0 456	0 317	4.825
0.83	0 498	0 301	4.838
0.84	0 539	0 284	4.850
0.85	0.580	0.267	4.862
0.86	0 622	0.249	4.875
0.87	0 666	0 232	4 887
0.88	0.708	0.215	4.899
0.89	0 741	0.197	4.911
0.90	0 793	0.180	4.924
0.91	0.836	0.162	4.936
0.92	0.878	0 145	4.948
0.93	0 922	0 127	4.961
0.94	0.966	0 109	4.973
0.95	1.010	0.091	4.985
0.96	1.053	0 073	4.998
0.97	1.098	0.055	5.010
0.98	1.142	0.036	5.022
0.99	1.185	0.018	5.035
1.00	1.232	0.000	5.047

Basal Metabolism. The respiratory exchange and, hence, the heat production of an individual may vary within wide limits, being influenced by such factors as muscular activity, emotional stress, food intake,

[14] Lusk, "Science of Nutrition," 1928 edition, p. 65.

[15] McClendon and Medes, "Physical Chemistry in Biology and Medicine," p. 158.

and external temperature. The influence of these factors is reduced to a minimum when the individual is lying perfectly still, sufficiently long after the last meal, so that no digestion is going on, and at a temperature ranging between 30° and 35° C. This condition of minimum metabolism and heat production is called basal metabolism [16] (*Grundumsatz*—Magnus Levy). It is also referred to as maintenance metabolism (*Erhaltungsumsatz*—Loewy), post-absorptive metabolism (F. G. Benedict), fasting metabolism, basal metabolic rate (Plummer and Boothby), and standard metabolism (Krogh).

The instruments used clinically in the determination of basal metabolism are for the most part of the closed-circuit type and are portable. No attempt will be made here to describe these at length. With each instrument the manufacturers supply a description of the apparatus and explicit instructions for its use.

In preparing for the test the subject abstains from food and drink (except water), following the evening meal which precedes the morning on which it is to be performed. Exertion before the test is strictly avoided. The subject should be completely at ease, both physically and mentally. The basal metabolism is determined by measuring the volume of oxygen consumed during a short interval (usually 8 minutes, or somewhat longer), from which the consumption per minute may be calculated. No special chamber is required; the subject simply breathes in oxygen from a bell-type spirometer. The expired air passes through a tank containing soda lime, which absorbs the carbon dioxide. As the oxygen is used up, the spirometer falls, the drop being automatically recorded on a revolving chart cylinder in terms of cubic centimeters. From the oxygen consumption the basal metabolic rate may be computed by consulting the tables of data giving the proper corrections for temperature, barometric pressure, height, weight, age, and sex.

Relation of Energy Metabolism to Surface Area. It was pointed out by Voit[17] that the heat production, during rest, of such animals as the mouse, rabbit, fowl, dog, and horse, is dependent on the surface area. Calculated on this basis, the heat output of these animals, as well as of man, amounts roughly to about 1000 calories per square meter per day. In proportion to its weight, the mouse has a greater surface and a greater heat output than the horse. Rubner[18] postulated the law that the metabolism is proportional to the superficial area of an animal. In the nor-

[16] The terms "minimum" and "basal" are often misinterpreted. It should be realized that, even when all the conditions for the proper conduct of the test are met, the results of any series of determinations of basal metabolism that may be performed on a given subject will show some variation. To look upon the lowest value as representing the true basal metabolism for the subject is, however, erroneous. As has been brought out by Boothby, Berkson, and Dunn, *Am. J. Physiol.*, **116**, 468 (1936), "metabolism is a variable quantity that can be standardized, but not absolutely fixed."

[17] *Z. Biol.*, **41**, 120 (1901).

[18] Rubner, "Energiegesetze," 1902, p. 282.

mal male, between the ages of 20 and 40 years, the heat output per square meter per hour is, on an average, 39.5 calories; in females between these ages, it is somewhat lower, namely, 36.5–37 calories.

The surface area of human subjects may be calculated by means of the following formula, proposed by D. Du Bois and E. F. Du Bois: [19]

$$A = W^{0.425} \times H^{0.725} \times 71.84$$

where A = area in square centimeters, W = weight in kilograms, and H = height in centimeters.

Influence of Age and Sex. New-born infants have a low basal metabolism, as was clearly demonstrated by Hasselbach,[20] who found that the heat production per kilogram of body weight was about the same during the first 24 hours of life as in the adult, despite the relatively larger surface of the infant. These findings were confirmed by Benedict and Talbot,[21] who observed a caloric output per square meter per 24 hours of 612 calories or about 25 calories per square meter per hour; and by Murlin, Conklin, and Marsh,[22] who obtained a value of 700 calories in the new-born. It is interesting to note that during the first hours of life the respiratory quotient is high, often approximating 1, which means that the infant is burning carbohydrate, for the most part. The quotient falls rapidly, so that at the end of the first day it may approximate 0.7 to 0.72. It then increases gradually until the fifth or sixth day, when it reaches 0.81, or the respiratory quotient of the adult. Carpenter and Murlin [23] made the interesting observation that the energy metabolism of the pregnant mother, immediately before delivery, is equal to the sum of the metabolism of the mother and infant taken 3–10 days after childbirth.

This has been confirmed by Rowe and Boyd,[24] who determined the

[19] The surface area of human subjects may be determined more directly from a chart prepared by D. and E. F. Du Bois. For the methods employed in deriving the equation, consult the original papers by Du Bois (*Arch. Internal Med.*, **15**, 868 [1915]; *ibid.*, **17**, 863, 887 [1916]), and the book previously cited.

The surface area of fetuses and children has been investigated by R. E. Scammon and associates, *Proc. Soc. Exptl. Biol. Med.*, **27**, 445, 449, 456, 461, 463 (1929–30). A. G. Hogan and C. I. Skouby (*J. Agr. Research*, **25**, 419 [1923]) have developed formulas for the surface areas of swine and cattle. G. R. Cowgill and D. L. Drabkin have derived a formula for the surface area of dogs (*Am. J. Physiol.*, **81**, 36 [1927]).

For comparing the surface area of different animals various formulas relating the surface to $\frac{2}{3}$ power of the weight have been used by a number of writers (see F. G. Benedict, *Yale J. Biol. Med.*, **4**, 385 [1932]). For a discussion of the validity of this relationship, as well as for other data concerning the surface area of various animals, the reader is referred to the comprehensive review by S. Brody, *Ann. Rev. Biochem.*, **3**, 295 (1934).

[20] Trans. Pub. No. 233, Carnegie Inst., Wash., 1915.

[21] Carnegie Inst., Wash., 1921 Pub. No. 302; see also, Talbot, "Basal Metabolism of Children," *Physiol. Rev.*, **5**, 477 (1925).

[22] *Am. J. Diseases of Children*, **29**, 1 (1925).

[23] *Arch. Internal Med.*, **7**, 184 (1911).

[24] *J. Nutrition*, **5**, 551 (1932).

changes in metabolism in a series of 77 pregnant women. During the third and fourth months of gestation a rapid decline in basal metabolism was observed. This was followed during the last 6 lunar months by a steady rise amounting to 13 per cent, or more, in excess of that conditioned by the gross increase in body weight. Taking into consideration the constancy of heat output in terms of surface area when the fetal area was added to that of the mother, the conclusion was reached that the excess in metabolism was contributed primarily by the fetus.

That there is in pregnancy the added stimulus to metabolism due to increased thyroid function, especially in adolescents, is suggested from the work of Enright, Cole, and Hitchcock.[25]

The basal metabolism of premature infants is lower than that of full-term infants, both at birth and for several months thereafter.[26] The basal metabolism of the child increases very rapidly during the first year of life and continues to be high for three or four years thereafter (15–20 per cent per square meter of surface above adult). A second rise has been reported by Du Bois preceding puberty (twelfth to thirteenth year). This is said to reach a maximum when the menses are established in girls and sexual maturity in boys.[27] It is followed by a gradual decline after puberty is reached.

The detailed observations of Lewis, Kinsman, and Illif[28] have shown that, in contrast to the adult, metabolism in the child (age period investigated 2 to 12 years) is continuously changing. It was found that the following methods of expressing heat production yielded data showing least dispersion: (1) calories per hour referred to weight and surface area, respectively; (2) calories per hour per square meter, referred to age.

An equally exhaustive investigation on older children (12 to 20 years) by Talbott[29] disclosed a closer correlation of heat production with body weight than with any other factor. Since there is also a high degree of correlation between heat production and creatinine excretion and since the latter is assumed to be a measure of the amount of active protoplasmic tissue, a relation of the latter to energy metabolism is implied. According to Talbott's data, race was without influence on metabolism, but the geographical situation (degrees of latitude), which cannot be separated from climate, seemed to exert a definite modifying effect.

[25] *Am. J. Physiol.*, **113**, 221 (1935).

S. Brody and R. C. Procter, *Mo. Agr. Exp. Sta., Res. Bull.*, **176**, 40 (1932), measured the oxygen consumption of pregnant cattle, sheep, and swine. The rise in metabolism in the latter part of gestation in all these animals exceeded the increase that could be ascribed to the fetuses. Compare with F. G. Benedict and E. G. Ritzman, *Wiss. Arch. Landw. Abt. B*, **1**, 1 (1931); cited by S. Brody, *Ann. Rev. Biochem.*, **3**, 338 (1934).

[26] H. H. Gordon and S. Z. Levine, *Am. J. Diseases of Children*, **52**, 810 (1936).

[27] A. Topper and H. Mulier, *ibid.*, **43**, 327 (1932).

[28] *Ibid.*, **53**, *Part II*, 348 (1937).

[29] F. B Talbott, E. B. Wilson, and J. Worcester, *ibid.*, **53**, *Part II*, 275 (1937).

According to Boothby and Sandiford,[30] there is a decrease in the basal metabolism of male children between the ages of 5 and 21 and a more rapid decrease in female children between the ages of 5 and 17, followed in both sexes by a gradual and nearly parallel decline to old age. The influence on metabolism of age and sex is shown in Table LV.

TABLE LV

BASAL METABOLISM STANDARDS ACCORDING TO BOOTHBY AND SANDIFORD*

Calories per square meter per hour

Age	Males	Females	Age	Males	Females
5	(53 0)	(51 6)	20–24	41 0	36.9
6	52 7	50 7	25–29	40.3	36.6
7	52 0	49 3			
8	51 2	48 1	30–34	39 8	36.2
9	50 4	46 9	35–39	39 2	35 8
10	49 5	45 8	40–44	38 3	35.3
11	48 6	44 6	45–49	37 8	35 0
12	47 8	43 4	50–54	37 2	34 5
13	47 1	42 0	55–59	36.6	34 1
14	46 2	41 0			
15	45 3	39 6	60–64	36 0	33.8
16	44.7	38 5	65–69	35 3	33 4
17	43 7	37 4			
18	42 9	37 3	70–74	(34 8)	(32.8)
19	42 1	37 2	75–79	(34 2)	(32 3)

* For a more detailed tabulation see W M. Boothby, J. Berkson, and H. L. Dunn, *Am. J. Physiol.*, **116**, 468 (1936).

In women the highest basal metabolism is usually attained in the premenstrual period and the lowest during the menstrual days.[31] Accordingly these days are avoided in making the determination for diagnostic purposes.

In elderly persons the basal metabolism is remarkably constant from day to day, provided emotional disturbance is eliminated. According to Benedict,[32] the basal metabolism of women over 60 years of age is approximately 1000 calories per day and is essentially independent of size, excepting those who are either grossly overweight or underweight.

Climatic, Racial, Seasonal, and Other Variations. According to Gustafson and Benedict [33] metabolism tends to be at a low level in

[30] *Am. J. Physiol.*, **90**, 290 (1929).

[31] F. G. Benedict and M. D. Finn, *Am. J. Physiol.*, **86**, 59 (1928); F. A. Hitchcock and F. R. Wardwell, *J. Nutrition*, **2**, 203 (1929); F. R. Griffith and co-workers, *Am. J. Physiol.*, **87**, 602 (1929); C. J. Conklin and J. F. McClendon, *Arch. Internal Med.*, **45**, 125 (1930).

[32] *New Eng. J. Med.*, **212**, 1111 (1935).

[33] *Am. J. Physiol.*, **86**, 43 (1928).

winter and to rise to a higher level during the spring and summer. The factor of climate, apart from the racial factor, has not been clearly defined. That climate probably exerts some effect is indicated by the results of various comparisons that have been made in different regions of the United States. For example, Tilt [34] found the basal metabolism of young college women in Tallahassee, Florida (Lat. 30°), to be about 10 per cent below that of a similar group in Chicago, Illinois (Lat. 42°).[35] Essentially the same result was obtained by Coons [36] in her study of a group of college girls in Stillwater, Oklahoma (Lat. 36°). However, a survey of the habitual food intake of her subjects has convinced her that it is less than that of women in other sections of the United States and that this probably has an important bearing on their low basal metabolic rates.

Low values for white inhabitants of the Tropics have been reported by de Almeida [37] (Brazil), Sundstroem [38] (North Queensland, Australia), and others.

In the study of regional variation of basal metabolism, the racial factor has received most of the attention. Unfortunately many of the data that have been reported have been either unsubstantiated, or the subject of controversy. Nevertheless certain significant facts have been determined. Thus MacLeod, Crofts, and Benedict [39] found the metabolism of Oriental female students (7 Chinese and 2 Japanese) attending American colleges to be on the average 10 per cent below the standard for American women of the same age, despite the fact that all were living essentially under the same conditions. Necheles [40] found the basal metabolism of adult Chinese (20 to 30 years) to be equal to, or below, the lower limit of Westerners. In younger individuals (below 20 years) the metabolism was relatively higher than that of adult Chinese, perhaps even higher than that of Western youths. The lower metabolic rate generally observed in Orientals, Necheles has attributed to a greater degree of constant muscular relaxation. He also found that, unlike Caucasians, the Chinese do not show as marked a drop in basal metabolism during sleep, from which he has assumed that the average Chinese is nearly as relaxed when awake as when asleep.

Low metabolic rates have also been observed in Filipinos.[41] Contrary to the observations of others, studies on Japanese by Japanese

[34] *J. Biol. Chem.*, **86**, 635 (1930).
[35] K. Blunt, J. Tilt, L. McLaughlin, and K. B. Gunn, *ibid.*, **67**, 491 (1926).
[36] *Am. J. Physiol.*, **98**, 692, 698 (1931); C. M. Coons and A. T. Schiefelbusch, *J. Nutrition*, **5**, 459 (1932).
[37] *J. physiol. path. gén.*, **32**, 12 (1924).
[38] *Physiol. Rev.*, **7**, 320 (1927).
[39] *Am. J. Physiol.*, **73**, 449 (1925); *Proc. Natl. Acad. Sci., U. S.*, **11**, 342 (1926).
[40] *Chinese J. Physiol.*, **6**, 153, 201 (1932); *Am. J. Physiol.*, **91**, 661 (1930).
[41] M. Ocampo, N. Cordero, and I. Concepcion, *J. Nutrition*, **3**, 237 (1930–31).

investigators have yielded results which are approximately within the range of variation for Europeans and Americans.[42]

A series of investigations conducted by Benedict and associates [43] has shown that the male Mayas of Yucatan have a high basal metabolic rate, average about 6.5 per cent above the normal standard for whites.

Perhaps the highest values (average of 33 per cent above normal) have been reported for Eskimos living in the Baffin Bay region (Heinbecker).[44] The lowest appear to be those obtained by Wardlaw and Horsley [45] on full-blooded Australian aborigines (average of −30.7 below the Aub-Du Bois standard). That the factor of climate is only partly responsible is indicated by the fact that the white Australian in the same region has a basal metabolism which is only about 10 per cent below the normal standard.

Of interest too are the observations of Turner [46] on the basal metabolism of women in the Near East. In certain racial groups, such as the Armenians, the metabolism was found to be within the normal range of variation for Westerners. Typical Syrian women, however, were found to have a low metabolism (−12.1 per cent as compared with an average value of −7.1 per cent obtained in a small group of European and American women living in Syria who served as controls). The native well-to-do Syrian women habitually lead an inactive physical life. Four of the women were apparently not pure Syrian Arabs, being light-haired and blue-eyed, traits which are presumably an inheritance from the days of the Crusaders. These women manifested a basal metabolism which deviated little from the normal. A somewhat similar illustration of the possible effect of racial admixture on basal metabolism is given by Wardlaw and Horsley, who state that the offspring of Australian aborigines, crossed with the white stock, have approximately the same metabolism which characterizes the white inhabitants of the district.

Very low values have also been reported for female Tamils and Malayalis, natives of South India.[47]

It is therefore evident that race is probably a specific factor, but that other influences are superimposed on this is also apparent. Thus, the high metabolism of Eskimos is not only a racial characteristic, but may be related to climatic conditions and the mode of life—strenuous phys-

[42] S. Okada and associates, *Arch. Internal Med.*, **38**, 590 (1926); also Takahira, cited by Du Bois, *J. Nutrition*, **3**, 217, 331 (1930–31).

[43] G. D. Williams and F. G. Benedict, *Am. J. Physiol.*, **85**, 634 (1928); G. C. Shattuck and F. G. Benedict, **96**, 518 (1931); M. Steggerda and Benedict, **100**, 274 (1932).

[44] *J. Biol. Chem.*, **80**, 461 (1928); see also I. M. Rabinowitch and F. C. Smith, *J. Nutrition*, **12**, 337 (1936).

[45] *Australian J. Exptl. Biol. Med. Sci.*, **5**, 263 (1928).

[46] *J. Am. Med. Assoc.*, **87**, 2052 (1926); E. L. Turner and E. Aboushadid, *Am. J. Physiol.*, **92**, 189 (1930).

[47] E. D. Mason and F. G. Benedict, *Indian J. Med. Research*, **19**, 75 (1931).

ical activity, a high meat diet, good physical development, increased muscle tone, etc. On the contrary, the low metabolism of the Australian aborigines may be associated with the warm climate, their lethargic habit of life, poor physical development, accompanying a chronic state of undernutrition.

The factor of diet is probably significant, although on this point also unequivocal data are lacking. Prolonged vegetarianism (five years, or longer) has been associated with a low basal metabolic rate by Wakeham and Hansen.[48] McClellan and associates[49] have studied the effect of an exclusive meat diet in two individuals. During the first few weeks a definite increase in metabolism was noted, but at the end of a year on this diet the metabolism was practically the same as at the beginning of the experiment.

Mental State. Apprehension, the anticipation of physical pain, tenseness, or an overactive, talkative, elated mental state tend to accelerated metabolism; neuroses, associated with a depressed mental state, are often accompanied by a somewhat reduced basal metabolism.

Starvation. Turning to the abnormal variations of the basal metabolic rate, we may consider first the effect of undernutrition and starvation. In a celebrated experiment, Benedict[50] studied the changes in basal metabolism of a man subjected to a fast of 31 days. At the beginning of the experiment the subject weighed 60.64 kg.; on the thirty-first day the weight was 47.39 kg. On the first day of the fast the heat output was 904 calories per square meter of body surface. By the twenty-first day this had diminished to 664 calories. Then followed an increase to 737 calories on the last day of the experiment.

Horst, Mendel, and Benedict[51] have made a study of the metabolism of albino rats during prolonged fasting at two different environmental temperatures, namely 16° and 26° C. At the beginning of the fast, the average weight was 222 grams. The total metabolism of the individual rats kept at 16° was distinctly higher (about 80 per cent) than that of the animals kept at 26°, and while in the former group there was little change in metabolism (per square meter per 24 hours), in the latter group an average decline of 36 per cent was recorded on the seventh day. As this fact would suggest, the loss in weight was therefore more rapid in the group kept at the lower temperature. The animals fasting at 26° survived on an average 16.5 days and lost 49 per cent of their initial body weight, while the animals kept at 16° lost nearly as much weight (44 per cent) within a period of 11 days, this being the average survival time in this group. The respiratory quotient, within a few hours after feeding, varied from 0.8 to 0.89. Determinations made

[48] *J. Biol. Chem.*, **97**, 155 (1932).
[49] *Ibid.*, **93**, 419 (1931).
[50] Carnegie Inst. Pub., No. 203, 1915; *Am. J. Physiol.*, **41**, 292 (1916).
[51] *J. Nutrition*, **3**, 177 (1930–31); see also Benedict, Horst, and Mendel, *ibid.*, **5**, 581 (1932).

after a fasting interval of 20 hours yielded values that were in no instance above 0.75 and usually below this point, indicating that the glycogen reserves were almost completely withdrawn at this time, an observation agreeing with one previously reported by Cori and Cori.[52]

This effect of undernutrition has been studied in individuals who, voluntarily or otherwise, were victims of chronic inanition.[53] During the period of the Great War, Zuntz and Loewy [54] followed their own basal metabolism and observed reductions of 15 and 12 per cent, respectively. Benedict [55] placed a squad of athletic men, who had been accustomed to a daily caloric intake of 3200–3600 calories, on a diet containing 1400 calories for a period of three weeks. As a result, the men lost, on an average, 12 per cent of their weight. The basal metabolism was reduced 18 per cent. After this the men were able to maintain themselves, without further loss of weight, on 1950 calories, although on this reduced intake they were not as active or energetic as previously, nor were they able to withstand the cold as well. Clearly, undernourishment and starvation are effective in lowering basal metabolism.

Influence of Disease. In certain disorders metabolism is increased; in others it is diminished. The most conspicuous changes are seen in thyroid disease. Of a group of 452 patients with exophthalmic goiter studied by Boothby and Sandiford,[56] 92 per cent had a basal metabolism of 20 per cent, or more, above normal, and in 52 per cent the metabolism was 50 per cent, or more, above normal.[57] In very severe cases, values in excess of the normal by more than 75 per cent are not infrequently encountered.

Contrasted with these high values are the low ones seen in hypothyroidism (myxedema, cretinism). Basal metabolic rates of 20 per cent below normal are the rule, and in rare instances the metabolism may be only 50 per cent of normal.

Basal metabolism is high in hyperpituitarism and low in hypopituitarism. Subnormal values are also associated with Addison's disease (adrenal insufficiency), nephrosis, and, as we have seen, in chronic undernutrition. Obesity due to endocrine dysfunction is usually accompanied by a low metabolism. A proportion of obese individuals, however, maintain a normal energy exchange on the basis of their surface area and hence an increased heat production, when computed on the basis of what should be their proper weight.

[52] *J. Biol. Chem.*, **70**, 557 (1926).

[53] The subject of fasting and undernutrition is discussed in a book of that name by S. Morgulis, Dutton & Co., New York, 1923.

[54] *Biochem. Z.*, **90**, 244 (1918).

[55] Benedict, Miles, Roth, and Smith, Carnegie Inst. Pub., No. 280 (1919).

[56] *Physiol. Rev.*, **4**, 69 (1924).

[57] These calculations are based on the Du Bois normal standards. Thus, if the normal basal metabolism of a woman for a given age is 37 calories per square meter per hour, and the actual metabolism is 44.4 calories, the metabolism is $44.4 - 37 = 7.4$; $7.4/37 \times 100 = +20$ per cent.

Apart from hyperthyroidism (Graves' disease, malignancy of the thyroid) and hyperpituitarism, the basal metabolism is increased in leukemia, polycythemia, occasionally in anemia, and in heart failure if dyspnea is present. Metabolism is also elevated in fever. An increase of 1 degree Centigrade in the body temperature causes a rise in metabolism of about 13 per cent. Thus, in typhoid fever there may be an increased heat production of as much as 40 or 50 per cent above the normal level. Similar changes are observed in pneumonia and malaria, but not in tuberculosis, where alterations in metabolism with changes in body temperature are not so marked. It is believed that toxic destruction of protein, which is characteristic of certain febrile conditions, is responsible for the increased metabolism. It is not definitely known whether the rise in temperature in fever is the cause or the result of increased tissue catabolism.

Regulation of Body Temperature. Animals may be divided into two groups according to their ability to maintain a constant body temperature. The temperature of cold-blooded animals varies with the environment, and they are therefore called heterothermic or poikilothermic. Reptiles, molluscs, fish, and insects belong to this group. At low temperatures (1–20° C.), these animals are usually warmer than their environment by about 1° C. (Burns [58]). When the temperature of the environment of a frog is increased to about 40° C., its own temperature remains somewhat lower.

Birds and mammals belong to the group of homoiothermic animals, which are able to resist environmental temperature changes. It is not to be supposed, however, that the mechanism for the maintenance of body temperature in these animals is never broken down. Reference has already been made to the body-temperature changes which occur during fever. During hibernation, the homoiothermic animal is essentially heterothermic. Curare produces a similar effect. This drug inhibits the transmission of motor impulses to voluntary muscles and causes, in addition, the breakdown of the temperature-regulating mechanism (Roehrig and Zuntz [59]). Its administration is also followed, at ordinary temperatures, by a marked diminution in metabolism. It is a matter of general knowledge that the regulation of body temperature is deficient in infants as well as in other new-born warm-blooded animals. These are usually unable to withstand long exposure to temperatures below 20° C.

Judging from experiments on rabbits, the temperature-regulating apparatus is located in the diencephalon.

The temperature of birds varies with the size and is usually between 40° and 43° C. Small mammals have a higher body temperature (39°-

[58] D. Burns, "An Introduction to Biophysics," London, 1921, Chapter XXXI.

[59] *Arch. ges. Physiol.*, **4**, 57 (1871). For a readable account of the mechanisms involved in maintaining constancy of body temperature, see W. B. Cannon, "Wisdom of the Body," W. W. Norton & Co., New York, 1932, Chapter XVI.

40° C.) than larger mammals. In man, the temperature is normally about 37.5° C., but may fall somewhat below 37° during sleep. The body temperature of the elephant is about 36° C.

Loss of Heat from the Body. Heat is lost from the body through the following channels:

1. Conduction and convection.
2. In warming the inspired air (conduction).
3. In warming the ingested food.
4. In excreta (CO_2, urine and feces are warm).
5. Radiation.
6. Evaporation of water from lungs and skin.

Among the factors that influence the dissipation of heat from the body are the area and moistness of the surface, time of exposure, temperature gradient between the surface and outside atmosphere, humidity of the atmosphere, and the force of the wind, if any. The loss of heat by radiation is affected by the color of the surface. A black surface has a higher absorptive and emissive power than a white surface. Accordingly, there should be a greater amount of heat lost by radiation from the body of a negro than from that of a white person. However, Hardy [60] has submitted evidence that all skin, irrespective of the visible color, radiates like a black body. But on the whole the loss of heat by radiation is comparatively small. In Table LVI (taken from Burns [58]) is given a rough estimate of the amount of heat lost per day through the more important channels:

TABLE LVI

LOSS OF HEAT FROM THE BODY

	Per Cent	Calories per Day
1. Radiation and conduction	73.0	1792
2. Evaporation		
(*a*) Lungs, etc.	7.2	182
(*b*) Skin	14.5	364
3. Excreta:		
(*a*) CO_2	3 5	84
(*b*) Urine and feces	1.8	48
Total heat loss per day	.....	2470

Having considered the way in which the accumulation of heat is prevented, we may now turn our attention to the factors which prevent

[60] *J. Clin. Investigation*, **13**, 615 (1934); J. D. Hardy and C. Muschenheim, *ibid.*, **13**, 817 (1934). Compare with S. Christiansen and T. Larsen, *Compt. rend. soc. biol.*, **118**, 921 (1935); **121**, 1230 (1936).

the excessive loss of heat from the body when the temperature of the environment is reduced. These are usually discussed under two heads, namely:

1. Physical regulation.
2. Chemical regulation.

Regulation of the temperature by physical forces is believed to predominate above 20° C. Below this temperature, chemical regulation enters into play to a greater degree than physical regulation.

Physical Regulation. Man adjusts the temperature of his environment by living in houses, by heating his dwelling during the winter, and by wearing clothes. Animals, likewise, provide themselves with shelter and have fur and feathers to enable them to diminish the loss of heat by conduction. The thickness of the skin and the amount of subcutaneous fat are additional factors which reduce loss of heat from the body. It is well known that lean people suffer more from cold and less from heat than obese individuals.

Heat and cold exert an important effect on the cutaneous nerve endings, causing a reflex vasomotor stimulation. When the temperature is high, the blood vessels of the skin and respiratory passages become dilated; there is an increased flow of blood to these areas, and hence a greater effective cooling surface, together with increased evaporation of water from sweat glands and mucous surfaces. On the other hand, cold, by causing vasoconstriction, decreases the flow of blood to the skin and respiratory surfaces and therefore diminishes the cooling area as well as the amount of perspiration.

In sleeping, an animal will curl up when it is cold and stretch out when hot, thereby diminishing or increasing the area of the exposed surface.

Chemical Regulation. The increased heat production incident to exposure to cold is referred to as the heat of chemical regulation. Opinion is divided as to its cause. Voit [61] suggested the view that exposure of the skin to cold brought about a reflex stimulation of metabolism in muscle cells without necessarily causing muscular activity such as shivering. From the work of Loewy [62] and others, however, it appears that the increased heat output is to be attributed to involuntary muscular activity. Shivering in man begins when the skin attains a temperature of approximately 19° C. (Swift).[63] During the period of shivering which follows immersion in a cold bath, the heat production may increase to 180 per cent above the normal (Lusk). In the series of observations by Swift on human subjects in a basal condition, exposed to an environmental temperature of about 2° C., the metabolism was found to increase as much as 400 per cent during the period of intense

[61] *Z. Biol.*, **14**, 80 (1878).
[62] *Arch. ges. Physiol.*, **46**, 189 (1890).
[63] *J. Nutrition*, **5**, 213, 227 (1932).

shivering. The energy required for involuntary muscular activity is derived from the metabolism in the tissues. It is well known that exposure to cold is an effective method of depleting the glycogen supply of the body, but that this is not necessarily due to the preferential oxidation of carbohydrate has been suggested by Swift. The probable importance of fat, as well as carbohydrate, is indicated by two results: (1) there is no change in the protein metabolism corresponding to the increase in energy metabolism; (2) the reaction to cold in a general way varies inversely as the amount of subcutaneous fat.

Continued exposure to cold stimulates the thyroid, which in turn raises the level of metabolism. Animals with thyroid deficiency have a low resistance to cold. The adrenals and pituitary also appear to be of importance in temperature regulation.

Shivering may be avoided by voluntary muscular exercise, for in either case the effect on combustion in the tissues is the same. When the difference in temperature between the body and the surrounding medium becomes so great that the dissipation of heat from the former is markedly increased, the " fires of metabolism " are caused to burn more briskly in order to make up for this loss. The chemical regulation of body temperature is thus essentially the result of increased metabolism.

Influence of Food on Metabolism; Specific Dynamic Action. For a variable period (usually 12 to 18 hours) after the ingestion of food, the calorific output is greater than that determined under basal conditions. To illustrate what is meant, let us suppose that the basal metabolism of a given individual is found to be 1800 calories per day. If exactly 1800 calories were now supplied to this individual in the form of a mixed diet, over a period of 24 hours, his heat production for that period would be, not 1800 calories, but more nearly 2000 calories. The problem we have to consider is the cause of this increased heat production.[64]

It is conceivable that the processes of digestion and absorption may constitute a factor; but when this supposition is tested experimentally, it is found that the energy requirement due to increased activity of the muscles and glands of the alimentary tract accounts for but a fraction of the increased heat production. Meat extract, though stimulating the digestive glands to secretion, nevertheless produces no appreciable effect on metabolism; nor does the presence of agar in the alimentary canal influence the rate of metabolism, although it probably increases the muscular activity of the intestinal wall. Moreover, it has been shown that the intravenous injection of glucose and amino acids leads

[64] Under certain conditions this effect is not apparent, as in the experiment of Richardson and Mason, *J. Biol. Chem.*, **57**, 587 (1923). These investigators calculated the amount of protein, fat, and carbohydrate utilized by a fasting subject during a period of 24 hours. On the basis of these calculations, a food mixture was prepared and fed in small amounts every 2 hours. This "replacement" diet caused practically no increase in basal heat production.

to an increase in metabolism which is almost comparable to that observed following the administration of these substances by mouth (Benedict and Carpenter [65]).

The effect of foods in stimulating metabolism is called the *specific dynamic action*, a term originated by Rubner.[66] This investigator supposed that the fundamental metabolism of a normal warm-blooded animal was always constant. The intake of food raised the metabolism, presumably because of intermediary reactions which had nothing to do with the fundamental energy requirement. Protein, according to Rubner, exerted a specific heat-raising effect. In the main, these principles were at one time accepted by Lusk,[67] who, with his students, contributed much to our knowledge of metabolism. In his attempt to define more precisely the reactions concerned in the production of the extra heat, considerable evidence was submitted in support of the idea that the specific dynamic action of proteins and amino acids was related to the conversion of the deaminized residues of the amino acids into glucose and glycogen.

Some of the earlier views of the fundamental cause of specific dynamic action have been abandoned: others are in the process of being abandoned—or at least of being radically revised. First, it may be noted again that the specific dynamic action is not influenced by the work of the digestive glands, intestinal movements, work of absorption, etc. In fact, this idea was rejected by Voit as far back as 1881. More recent experiments have shown that amino acids exert essentially the same effect whether administered orally, subcutaneously, or intravenously.

Secondly, specific dynamic action is not due to the direct combustion of amino acids. The early experiments of Lusk showed that in the phlorhizinized dog alanine and glycine were completely recovered in the urine as glucose; yet these amino acids produced their usual effect in raising metabolism.

Another explanation that has been set aside by various writers (e.g., Wilhelmj [68]) is that specific dynamic action is due to direct stimulation of the tissues by amino acids. Evidence opposed to this view has been submitted by Wilhelmj, Bollman, and Mann,[69] who found that, in the hepatectomized dog, the administration of glycine or alanine caused no rise in metabolism. It has been shown by Dock [70] that, after feeding rats on casein, the oxygen consumption of the hind quarters increased very little compared to the very striking elevation (141 per cent rise) which occurred in the viscera. From these experiments Dock

[65] Carnegie Inst. Wash., Pub. No. 261, 1918.

[66] M. Rubner, "Die Gesetze des Energieverbrauchs bei der Ernährung," Leipzig, 1902.

[67] G. Lusk, "Science of Nutrition," 1928 edition, p. 276.

[68] *Physiol Rev.*, **15**, 202 (1935).

[69] *Am. J. Physiol.*, **87**, 497 (1928).

[70] *Ibid.*, **97**, 117 (1931).

concluded that the liver, and not the muscles, is the site of the calorigenic effect of protein. This view is not shared by Bornstein and Roese.[71]

Lusk looked upon the non-nitrogenous residue of amino acids as the source of the specific dynamic effect, but more recently the extra heat production has been linked with the metabolism of the nitrogenous part of these substances. It is relevant to note that the administration of ammonium salts has been found to cause a marked rise in heat production. In fact, the two reactions which are thought to contribute largely to the calorigenic effect of amino acids are:

1. Oxidative deamination.
2. Synthesis of urea from ammonia.

The first of these reactions, according to Borsook,[72] contributes 4 calories per gram of nitrogen metabolized. A maximum of 4 calories per gram of nitrogen, but probably less, is assigned to the synthesis of urea from ammonia. Formerly, Borsook thought that the energy involved in the excretion of nitrogenous constituents by the kidney was of major importance; now he believes that at most 1–2 calories can be related to renal function. Altogether, about 7–10 calories are derived per gram of nitrogen (6.25 grams protein) metabolized. Borsook believes that this energy yield is the more constant of the two factors constituting the specific dynamic action. The second factor—more variable, and at times the larger fraction—arises from the non-nitrogen residues.

Concerning the specific dynamic action of carbohydrate and fat the older views have remained essentially unchallenged. It is significant that, in hepatectomized dogs, glucose exerts a higher specific dynamic effect than normally. This has been ascribed to the fact that in the absence of the liver none of the glucose is removed; therefore it all reaches the tissues in which it causes an elevation in heat production.

Williams, Riche, and Lusk [72] showed an increase of 30 calories in heat production for every 100 calories contained in the protein of 1220 grams of meat given to a dog. Numerous similar observations place the value for the specific dynamic action of protein at about 30 per cent, and this is approximately the same whether food protein or body protein is being metabolized. In the case of fat, Rubner thought it to be about 12 per cent; and of carbohydrate, 5 to 6 per cent. The value for fat has been revised, however, to approximately 4 per cent by Murlin and Lusk.[73]

Among amino acids, phenylalanine has the greatest specific dynamic action. Other amino acids which produce a conspicuous effect are glycine, alanine, leucine, and tyrosine.

It was realized by Rubner that the energy due to the specific dynamic

[71] *Pflüger's Arch.*, **223**, 498 (1930).

[72] *J. Biol. Chem.*, **12**, 371 (1912); cited by Lusk, "Science of Nutrition," 1928 edition, p. 283; *Ergeb. Physiol.*, **33**, 103 (1931).

[73] *J. Biol. Chem.*, **22**, 15 (1915); see also J. R. Murlin, A. C. Burton, and W. M. Barrows, *J. Nutrition*, **12**, 613 (1936).

action of foodstuffs was not available to the organism for physiological work, but that it could be utilized in the chemical regulation of body temperature. At low temperatures, 0–5° C., the basal (fasting) energy production is already near the resting maximum; hence no increase follows the intake and metabolism of protein. At somewhat higher temperatures (15°–20°) the specific dynamic action is due almost exclusively to protein (deamination, urea formation, nitrogen excretion); no excess heat is liberated due to carbohydrate or fat. It is only when the environmental temperature has reached 25°, or above, that carbohydrate, fat, and the deaminized residues contribute to the total calorigenic effect. At these higher temperatures, " chemical " regulation is inoperative; the body temperature is now governed by the physical heat-regulating mechanisms.

Several writers have recognized that there is in reality little basis for Rubner's terminology, since the increased heat production which follows the intake of foods is the result of several processes rather than of one specific process. There is also the question of whether the use of the word " dynamic " is appropriate in this connection. The idea is perhaps better conveyed by the term " *calorigenic action.*" [74]

Influence of Work on Metabolism. Work is accomplished by the body at the expense of increased metabolism, whether food is eaten or not. In a series of experiments, Rubner [75] was able to show that heat production incident to mechanical work is independent of the heat produced because of specific dynamic action, especially in the case of protein food. This is indicated by the data in Table LVII.

TABLE LVII

INFLUENCE OF DIET AND MECHANICAL WORK ON METABOLISM

Diet and Conditions	Calories Produced		
	24 Hours, Calories	Increase, Per Cent	Increase Due to Work, Calories
No food, rest	1976		
Cane sugar 600 g. + rest	2023	2 4	
Same + work (100,000 kg-meters)	2868	45.2	845
Protein (meat) + rest	2515	27 2	
Protein + work (100,000 kg-meters)	3370	70 5	855

However, Rapport [76] has presented evidence that the specific dyanmic action of fat, as well as of glucose, is abolished during muscular exercise.

[74] M. Kleiber, *Ann. Rev. Biochem.*, **6**, 375 (1937).
[75] *Sitzber. preuss. Akad. Wiss.*, **16**, 316 (1910); cited by Lusk, p. 409.
[76] *Am. J. Physiol.*, **91**, 238 (1929–30).

in short, that the extra energy which at rest appears as waste heat is utilized as free energy in muscular work. In experiments with protein, the specific dynamic effect was not abolished. Nevertheless these observations, if confirmed, constitute a distinct departure from Rubner's fundamental concept.

Although, fundamentally, there is little similarity between the animal body and the steam or gasoline engine, it is nevertheless of interest to compare the efficiency of external muscular work with the thermal efficiencies of mechanical devices. The efficiency of the steam engine is 8–10 per cent. The usual type of gasoline motors have an efficiency of about 20 per cent; i.e., of every 100 gallons of gasoline which are burned to completion, about 20 are converted into mechanical energy. Higher values (40 per cent) have been recorded for the Diesel engine. In man, it has been estimated that the maximum gross efficiency of muscular work, such as pulling a heavy load, is about 25 per cent.[77] This means that, of 100 calories of total energy expended during hard work, a maximum of 25 calories may be recovered as work accomplished, while the remaining 75 calories of expended energy is dissipated in various ways (basal metabolism, heat increment of feeding, standing above basal, walking without load above standing, overcoming internal resistance of body, useless incidental movements associated with pulling loads, " recovery " process—removing lactic acid resulting from work). In a well-conceived series of experiments Brody and Cunningham [77] found that the energy efficiency of muscular work is independent of the size of the animal; it was essentially the same for a 1500-pound Percheron gelding or a 600-pound Shetland pony as for a 150-pound man.

Training is an important factor determining the amount of energy required in the performance of a given task. An individual unaccustomed to a certain type of muscular exertion, such as mountain climbing, uses proportionately more energy than one who has been trained for this work. It has also been determined that the speed with which muscular work is done influences the degree of energy expenditure. Less energy is used in covering a given distance by slow walking than in covering it by fast walking or running.

[77] Gross, or overall, efficiency of muscular work may be expressed by the following formula (Brody and Cunningham):

$$\text{Gross efficiency} = \frac{\text{energy equivalent of mechanical work accomplished}}{\text{total energy expended while accomplishing work}}$$

The "*net*" efficiency is equivalent to

$$\frac{\text{Work done}}{\text{total energy used} - \text{energy of standing animal}}$$

The "absolute" efficiency is the

$$\frac{\text{Work done}}{\text{total energy used} - \text{energy of walking animal without load}}$$

Research Bulletin **244**, *Univ. Mo. Agr. Expt. Sta.*; see also S. Brody, *Ann. Rev. Biochem.*, **3**, 295 (1934); M. Kleiber, *ibid.*, **6**, 375 (1937); W. O. Fenn, *Am. J. Physiol.* **92**, 583 (1930).

Provided an adequate amount of fat and carbohydrate is available, muscular exercise does not influence materially the amount of protein metabolism. In the well-nourished individual, violent exertion is associated with a high respiratory quotient without marked alteration in nitrogen excretion, showing that carbohydrate is the chief fuel under these circumstances.[78]

Heat produced in doing mechanical work can take the place of heat of chemical regulation. In other words, there is not a summation of these factors when an individual performing muscular exercise is exposed to cold.

It has been determined by Benedict and Benedict[79] that intense mental effort, such as is entailed, for example, in solving mathematical problems (mental arithmetic), is accompanied by a relatively small increase in oxygen consumption and heat production. Even after an hour of sustained mental work, the average increase in metabolism in Benedict's experiment was only about 4 per cent, most, if not all of which was presumably due to incidental factors (increased muscular activity associated with increased ventilation and heart rate of the subjects). Benedict and Benedict have therefore concluded that mental effort, *per se*, is without significant influence on the energy metabolism.

Caloric Requirements. Rubner developed the concept that, under certain conditions, the foodstuffs may replace one another in accordance with their heat-producing value. This is known as the *isodynamic law*. According to this view, 100 grams of fat, 232 grams of starch, and 243 grams of protein (as dried meat, etc.) are equally effective in providing the animal body with the energy required for muscular work as well as with heat. Rubner's hypothesis has been questioned by Cathcart,[80] who believes that glucose and fat are not interchangeable in providing energy demands, since carbohydrate is a more effective protein-sparer than fat. Evidence has been presented, likewise, by Christensen,[81] Gemmill,[82] and others, which shows that carbohydrate is superior to other foodstuffs in supplying energy during hard work. Accordingly, the isodynamic law is not to be interpreted too strictly. As pointed out by Cathcart, the calorific value is simply a very convenient physical standard for the assessment of diets; but the mere fact that such a standard has proved of great utilitarian value is not a real justification for adopting it as the foundation stone of hypotheses framed to offer an

[78] Compare with D. Rapport and A. Canzanelli, *ibid.*, **101**, 85 (1932).

In this and other connections the student is referred to a review "The Fuel of Muscular Activity of Man," by T. M. Carpenter, *J. Nutrition*, **4**, 281 (1931). Consult also D. B. Dill, "The Economy of Muscular Exercise," *Physiol. Rev.*, **16**, 263 (1936).

[79] F. G. Benedict and C. G. Benedict, Publication 446, Carnegie Institution of Washington, 1933. See also Editorial, *J. Am. Med. Assoc.*, **102**, 540 (1934).

[80] *Biochem. J.*, **16**, 747 (1922).

[81] Series of papers in *Arbeitsphysiol.*, Vols. **4**, **5**, **7**; cited by Dill.[78]

[82] *Am. J. Physiol.*, **108**, 55 (1934).

explanation for cellular activity. The calorific value of a given amount of food is therefore not necessarily a criterion of its nutritional or tissue-sparing effect. With this in mind, we may continue our discussion of the caloric requirements of the animal body.

The caloric needs of man and animals are determined by the total heat production due to the various factors which have been discussed in the preceding paragraphs. When the caloric intake is equivalent to the output, the condition of calorific balance or equilibrium is said to exist. This is the normal state in the adult individual; but in the growing animal the intake should exceed the outgo by a considerable margin. The energy of maintenance, including that of the vital functions (circulation, respiration, secretion, excretion, maintenance of muscle tone, etc.), is represented by the basal heat production. The basal metabolism of an adult, weighing 70 kg., is approximately 1750 calories for 24 hours. The heat production is increased above the basal level even by slight activity, such as sitting or standing. Depending on the character of the diet, allowance should be made for the specific dynamic action of the food ingested. An allowance of 10–12 per cent above the basal metabolic requirement is ordinarily sufficient when the individual is maintained on a mixed diet.

The most variable factor to be reckoned with is the food required for the performance of physical work. The relation of occupation to energy requirement has been the subject of numerous investigations both in this country and abroad. Individuals engaged in sedentary occupations have a total daily metabolism of 2500–2800 calories. These figures allow 550–900 calories for the performance of mechanical work (walking to and from work, etc.).

The food consumption of the average American farmer is about 3500–4000 calories. Interesting observations on Hungarian peasants during harvesting have been recorded by Farkas.[83] He estimated the daily energy expenditure to be about 6000 calories. As the daily food consumption was, as a rule, equivalent to only 4800 calories, the peasants regularly lost weight during this season. Lusk states that a bicyclist riding for 16 hours may have a metabolism amounting to 9000 calories daily. The food ration of a Maine lumberman may rise to 7000 and even 8000 calories per day.[84]

In Finland, Becker and Hämäläinen [85] made a study of the energy requirements of men and women engaged in various occupations. A portion of their results is summarized in Table LVIII.

Thus far, we have not considered the allowance to be made for

[83] *Arbeitsphysiol.*, **1**, 466 (1929); **5**, 434, 549, 569 (1932); cited by S. Brody, *Ann. Rev. Biochem.*, **3**, 355 (1934).

[84] A critical discussion entitled "Some of the Difficulties in the Quantitative Assessment of Human Diets," by E. P. Cathcart, *Nutrition Abstracts & Revs.*, **1**, 6 (1931–32), will be found instructive. See especially the recent review by J. B. Orr and I. Leitch, *ibid.*, **7**, 17 (1938).

[85] *Skand. Arch. Physiol.*, **31**, 198 (1914).

TABLE LVIII

Sex	Occupation	Calorific Requirement for 24 Hours
Men	Tailors (2)	2400 to 2500
	Bookbinder	2700
	Shoemaker	2800
	Metal workers (2)	3100 and 3200
	Painters (2)	3200 and 3300
	Carpenters (2)	3200 and 3300
	Stonemasons (2)	4300 and 4700
	Men sawing wood (2)	5000 and 5400
Women	Seamstress (needle)	1800
	Seamstress (machine)	1900 and 2100
	Household servants	2300 to 2900
	Washerwomen (2)	2600 and 3400

"chemical regulation." This factor would become operative only when an individual doing little or no work is exposed to extreme cold, a combination of circumstances not ordinarily met with. In considering the quantitative relation between work and total metabolism, it is important to bear in mind the interplay of all the factors. The heat due to mechanical work can replace the heat of chemical regulation. Demands for chemical regulation of body temperature may be met, likewise, by the specific dynamic action of foodstuffs. On the other hand, it is generally held that none of the energy of specific dynamic action of foodstuffs can be converted into muscular work.

It is a mistaken idea that a child is a fraction of an adult as far as its food requirements are concerned. There are three points to be considered in this connection, namely, the relatively high basal metabolic rate during childhood, the usual physical activity of boys and girls, and the necessity of maintaining the caloric intake well above the expenditure in order to allow for growth. Between the ages of 1 and 2 years, infants require approximately 1000–1200 calories per day. It has been estimated that between the ages of 10 and 13 years, the requirements of a boy are 2300–3000 calories, and even more for a very active child. This explains the apparent voraciousness of boys and girls, particularly the former. That there is actually a physiological need for large quantities of food is shown in the work of several authorities,[86] all of whom recommend a most liberal food allowance for the growing child.

[86] The problem of energy requirements of children is discussed in the contribution of F. G. Benedict and Talbot, Carnegie Inst. of Washington, Publication No. 302 (1921), and in the monograph of Mendel, "Nutrition—The Chemistry of Life," Yale University Press, 1923; see also S. Z. Levine, *Am. J. Diseases of Children*, **50**, 596 (1935). A valuable summary is also to be found in the "Report of the Committee on Growth and Development, White House Conference on Child Health and Protection," Part III, Nutrition, Century Co., New York, 1932, pp. 334–424.

CHAPTER XVIII

NUTRITION

Sir Michael Foster likened the growth of knowledge to the ascent of a spiral stair from which the observer periodically surveys the same landscape, but each time from a higher level than the last.—JOSEPH BARCROFT.

The chemistry of nutrition is conventionally treated from the standpoints of (*a*) caloric, (*b*) mineral, (*c*) protein, and (*d*) vitamin requirements. These are generally regarded as the most important factors to be considered. There is, however, some evidence that although the body is capable of synthesizing fat from carbohydrate and indirectly from protein, nevertheless, a certain amount of it, present in the food as such, is indispensable to proper nutrition. The water balance of the organism, as has been pointed out in other connections, is another factor to be considered, being of special importance in the young. When deprived of sufficient water, animals develop complete anorexia.[1]

It is only in recent years that scientific research has revealed the intricacies of the problems of nutrition and the relation of certain diseases to specific dietary deficiencies. We have also just come to the realization that human experience and tradition cannot be relied upon invariably as a guide to proper nourishment. In the words of an eminent authority, Sir Frederick Gowland Hopkins:[2]

> It is often felt that concerning matters so urgent as its own nutrition, humanity, with all the experience of the ages behind it, can have little to learn from modern science, yet, as in the case of so many other established traditions, an assumption of this kind is wholly unjustified. Tradition accumulates prejudices quite as often as truths, and the former are apt to be more potent in their influence. With sufficient space it would be easy to show that faulty nutrition has played a large part in inhibiting human progress, and even to show that few races have at any time been ideally nourished.

To be adequate, a diet must provide for all the needs of the organism, particularly for maintenance, growth, and reproduction. The minimum requirements for proper nutrition are fulfilled only (*a*) when the organism is maintained in caloric equilibrium; (*b*) when it is maintained in nitrogen equilibrium; (*c*) when there is an adequate supply of inorganic elements; and (*d*) when there is an adequate supply of

[1] F. C. Bing and L. B. Mendel, *Am. J. Physiol.*, **98**, 169 (1931).
[2] *Nutrition Abstracts & Revs.*, **1**, 3 (1931–32).

vitamins. As can be readily appreciated, the needs of the young and growing animal are far in excess of the minimal requirements.

In addition to these factors, consideration may also be given to certain others which are presumably of secondary importance. There is some reason for believing that variety in the selection of food is beneficial. The Eskimo is limited by his environment to a few staples and subsists in certain localities largely on fish and meat. The everyday food of the Oriental of the poorer class is rice with variable additions of vegetables and fish. Europeans and Americans seek a more varied diet. The benefits of this may be purely psychological; but it is reasonable to suppose that the larger the variety of foods, the less would be the likelihood of missing some essential ingredient.[3]

There is also the question of cooking, seasoning, and flavoring. Aside from the effect of cooking in increasing the digestibility of many substances, the beneficial effect of these treatments is due largely to the increased palatability, and, hence, to increased consumption of food. Moreover, seasoning and flavoring materials are not without influence in stimulating the secretion of digestive juices (both chemical and psychic stimulation). Although these substances can hardly be regarded as essential to nutrition, except in isolated instances, perhaps, they may nevertheless be included here as constituting a factor of secondary importance. It may also be pointed out, in this connection, that diet is too often a matter of habit. Certain individuals and peoples relish foods that others find distasteful.

Caloric Requirements. A large portion of the preceding chapter was devoted to the energy factor in nutrition. The subject may therefore be dismissed here with a few additional words. The normal adult requires just sufficient calories to balance the total loss from his body. It is obvious that the manual laborer needs more calories than the individual who is engaged in light work. The caloric demands are also influenced by external temperature and, hence, by climate. When more calories are given off by an individual than are taken in the form of food, he is no longer in a state of caloric equilibrium. Calories are produced, under these circumstances, at the expense of the tissues, and there is a loss of weight. It is important to bear in mind that the growing child should be provided with more food than is sufficient for the maintenance of the energy balance. The normal state for the growing animal is a condition of positive caloric balance.

MINERAL REQUIREMENTS

The extraordinary influence of the mineral elements of nutrition, though appreciated to some degree for many years, was not clearly under-

[3] The dietary habits of man in different parts of the world are described by E. V. McCollum and N. Simmonds in "The Newer Knowledge of Nutrition," Macmillan & Co., New York, 4th edition (1929), Chapter XXVII.

stood nor quantitatively studied until quite recently. In a long series of experiments which began about thirty years ago, originally planned by Babcock and carried out by Hart, McCollum, and Steenbock,[4] the growth and reproduction of cattle upon restricted diets of various grains were studied. These investigators divided their experimental animals into four groups. All the animals received approximately the same amount of sodium chloride. The ration which was fed to one group was derived solely from the wheat plant and consisted of wheat straw, wheat gluten, and the entire wheat grain. The second group received a ration derived from the corn plant. The third group was fed on the products of the oat plant. The fourth group of animals received a ration consisting of a mixture of wheat, corn, and oats in about equal proportions. It was discovered that the nutritive condition of the corn-fed animals was much better than that of any of the remaining groups. The wheat-fed cattle fared worst. The corn-fed animals gave birth to normal young and reared them. The offspring of the wheat-fed cows were not carried to full term, and those that were not born dead usually died several days after birth. The behavior of the animals in the remaining two groups was intermediate between the two extremes observed in the corn-rationed and wheat-rationed groups. The untoward manifestations noted in the wheat-fed animals, as well as in those maintained exclusively on oats, were shown to be due largely to a defficiency in inorganic constituents, chiefly calcium.

Much of the progress attained in the field of nutrition has been made possible by using small animals, particularly albino rats. These reach maturity and begin to breed at about ninety days of age and rarely live to be more than three years old. Thus it is possible to study, in a comparatively short time, the complete life cycle of the animal. The use of synthetic or artificial diets in feeding experiments may likewise be mentioned in this connection. It is possible to maintain rats and other animals in excellent nutritive condition upon a diet consisting of purified protein, fat, carbohydrate, and salts, provided the necessary vitamins are added to the food. These may be derived from yeast, butter, cod-liver oil, and other sources. The value of employing suitable artificial mixtures in nutrition studies is obvious, for it becomes possible by this method to exclude, at will, more or less completely, one or more ingredients. It then remains only to compare the progress of the experimental animals in respect to growth, reproduction, etc., with control animals maintained on an adequate diet. Osborne and Mendel and their followers have done much to establish the concept, attributed to Liebig, that a deficiency of any factor essential for growth is followed by a failure in growth of the body as a whole, and not by the production of abnormal tissues due to the lack of some

[4] For details of these studies the student is referred to McCollum and Simmonds, "Newer Knowledge of Nutrition," as well as to Mendel's monograph on nutrition, Chapter II.

element. When the minimum requirements are not met, even with regard to a single constituent, such as a growth-promoting vitamin, an essential amino acid, or an inorganic element like calcium, failure in growth results.

Before considering the specific rôle of individual elements in nutrition, brief reference may be made to the effects resulting from a general mineral-salts impoverishment. It has been shown by Winters, Smith, and Mendel [5] that on diets low with respect to the inorganic constituents, rats cease to grow in weight, but continue to grow in length because of a persistent increment in the length of the long bones. An abnormal increase in the size of the kidney is another conspicuous finding. The increase in the weight of the long bones is largely accounted for by the higher content of organic residue (Smith and Schultz).[6] The spleen tends to diminish in size. The development of polycythemia is very striking and is especially marked when practically all the inorganic constituents are excluded from the diet. The erythrocytes become progressively smaller in size, but contain the normal percentage of hemoglobin; however, the absolute amount in each cell is diminished (Swanson and Smith).[7]

Sodium, Potassium, and Chloride. Osborne and Mendel [8] in an experimental study found the sodium and chloride requirements to be low. Normal growth occurred in rats on rations containing only 0.04 per cent (per cent of total food), or even less of each of these elements. The growth of the animals was fairly satisfactory on a potassium intake of about 0.03 per cent, provided that the sodium intake was adequate. When both sodium and potassium were reduced in amount, growth ceased. Under these circumstances, the addition of sodium alone, at an early stage of growth, did not result in gain; but when potassium was added, normal growth was resumed. At a later stage in development, Osborne and Mendel found it possible to replace sodium for potassium.

With regard to the potassium requirement, the observations of Miller [9] differ somewhat from the findings of Osborne and Mendel. Miller noted that the growth of rats could be greatly retarded by reducing the potassium content of the ration below a certain level, approximately 0.1 per cent. The minimal requirement, according to this author, is therefore at least three times as much as that given by Osborne and Mendel. Moreover, Miller observed that potassium deficiency during the early development of the organism may not only prevent the

[5] *Am. J. Physiol.*, **80**, 576 (1927); see also P. P. Swanson, C. A. Storvick, and A. H. Smith, *J. Biol. Chem.*, **114**, 309 (1936).

[6] *A. J. Physiol.*, **94**, 107 (1930); see also A. E. Light, P. K. Smith, A. H. Smith, and W. E. Anderson, *ibid.*, **107**, 681 (1934).

[7] *J. Biol. Chem.*, **98**, 479, 499 (1932).

[8] *Ibid.*, **34**, 131 (1918).

[9] *J. Biol. Chem.*, **55**, 61 (1923); **62**, 259 (1924); **70**, 587 (1926).

growth of the body but also cause abnormal physiological disturbances which make themselves apparent later. In fact, rats deprived of potassium early in life usually die despite an adequate supply of potassium at a later stage (fourth to eleventh week) of development. Miller did not obtain normal growth by substituting sodium for potassium. Nor is it possible to substitute potassium for sodium in a sodium-deficient diet (St. John).[10]

From the standpoint of adequacy in nutrition, no difficulties are encountered in the selection of diets containing sufficient amounts of sodium, chloride, and potassium. The last is especially abundant in both plant and animal tissues, and the quantities derived from these sources are far in excess of the normal requirements for proper nutrition.[11] Sodium and chloride are likewise widely distributed in nature. An adequate supply of these elements, particularly in the nutrition of man, is not difficult to secure, since the quantities of common salt used in seasoning are greater than the natural requirements. In herbivorous animals, however, particularly during lactation, there is occasional evidence of salt deficiency. It is well known that buffalo and deer frequently travel long distances and brave many dangers in search of rock-salt deposits, or salt licks. Observations reported by Babcock [12] have proved that the milk of cows deprived of salt may become very low with respect to the sodium chloride content and that continued deprivation may result even in the death of these animals. The old practice of supplying common salt to cattle therefore has a scientific basis.

An oft-repeated observation, first made by Bunge,[13] is that the administration of potassium increases the excretion of sodium and chloride in the urine. According to Whelan,[14] the increased elimination of these elements may be due to the diuretic effect of potassium.

The adrenal cortex, as we have seen (p. 489), is of importance in regulating salt metabolism. Among the conspicuous effects of adrenal insufficiency are a loss of sodium and chloride by excretion in the urine, a marked reduction of sodium chloride in the blood, and an increase in potassium. The rise in serum potassium seems to be more striking in the adrenalectomized animal (Hastings and Compere [15]) than in clinical adrenal insufficiency (Addison's disease). However that may be, the

[10] *J. Agr. Res.*, **37**, 55 (1928).

[11] Data submitted by J. Davidson and J. A. LeClerc (*J. Nutrition*, **11**, 55 [1936]), show that the mineral composition (sodium, potassium, chloride, calcium, magnesium, etc.) of vegetables obtained from different regions may vary considerably, being influenced by such factors as the kind of fertilizer treatment and the composition of the irrigation water.

[12] Wisconsin Agr. Exp. Sta. Ann. Report, 129 (1905); cited by McCollum and Simmonds, "The Newer Knowledge of Nutrition," 4th edition, 1929, p. 411.

[13] *Z. Biol.*, **9**, 104 (1873).

[14] *J. Biol. Chem.*, **63**, 585 (1925).

[15] *Proc. Soc. Exptl. Biol. Med.*, **28**, 376 (1931).

severe manifestations of adrenal insufficiency have been partly related to the combination of sodium chloride depletion and potassium retention. The symptoms of Addison's disease may often be ameliorated by the administration of sodium chloride and aggravated by the administration of potassium salts. Restriction of the potassium intake has therefore been advocated (Wilder and co-workers [16]). It has been estimated that, on a normal diet, the potassium intake is about 4 grams per day. The daily intake may be considerably reduced by avoiding or limiting the intake of certain foods rich in potassium.[17]

Calcium Requirement. In a study of a large number of American dietaries, Sherman [18] was led to the conclusion that the intake of calcium in this country is frequently below the level of requirement and that the adequate supply of this element in a "mixed diet" constitutes a real problem in human as well as in animal nutrition. From a study of calcium excretion in adults, Sherman determined that the minimum requirement of calcium is, on an average, about 0.45 gram per day (equivalent to 0.63 gram when expressed in terms of CaO). If a margin of safety of 50 per cent is allowed—a practice which has been found valuable in computing the needs for protein and other essential components of the diet—the so-called "standard requirement" of a normal adult of about 70-kg. weight would be 0.68 gram per day. The data given by Sherman show that 52 per cent of the dietaries studied were below this level, and that as many as 16 per cent were below even the minimal requirement of 0.45 gram.

The problem has also been reviewed comprehensively by Leitch,[19] whose estimate of the maintenance requirement for adults (0.55 gram calcium) is somewhat higher than Sherman's. In computing the gross requirement, it is assumed that only about 50 per cent of the intake is retained. On this basis, Leitch gives the following values (in grams of calcium) for the minimum gross requirement: from 6 months to 2 years 0.8, from 2 to 9 years 0.9, from 9 years 1 gram, increasing to 2 grams between 15 and 16 years. Thereafter the decrease is gradual to the adult maintenance requirement.

The effects of calcium deficiency are especially serious in children. In a study of calcium and phosphorus metabolism, Sherman and Hawley [20] have shown that children from 3 to 13 years old require an intake

[16] *Arch. Internal Med.*, **59**, 367 (1937).

[17] Potatoes are especially rich in potassium, containing more than 400 mg. per 100 grams. The greater part of the potassium (75–80 per cent) may be removed by boiling in several changes of water. The following foods are said to contain more than 300 mg. per cent of potassium: meats, fowl, fish of all kinds, potatoes, sweet potatoes, peas, beans, beets, cabbage, celery, chard, chestnuts, spinach and other greens, pumpkin, squash, parsnips, dried fruits (especially dates), figs, prunes, banana, pineapple, nuts of all kinds, wheat, bran and molasses (Wilder).

[18] *J. Biol. Chem.*, **44**, 21 (1920); see also *Harvey Lectures*, 1917–1919, p. 114.

[19] *Nutrition Abstracts & Revs.*, **6**, 553 (1937).

[20] *J. Biol. Chem.*, **53**, 375 (1922).

of 1 gram of calcium per day, an amount which is necessary to induce optimum storage of this element and to insure the proper development of bones and teeth. Milk is the best and most available source of calcium, particularly for children, who do not seem to utilize the calcium of vegetables very efficiently. McCluggage and Mendel [21] reported that the calcium supply of the organism is normally derived from milk in greater proportion than from any other dietary source and that the calcium in spinach and carrots is poorly assimilated.[22] Accordingly it has been concluded that vegetables should not be used with the idea of providing the requisite amount of calcium, or as a substitute for milk. However, it is not impossible for adults to meet the maintenance needs of calcium, as well as phosphorus, from exclusively vegetable sources, such as carrots, as has been shown by M. S. Rose,[23] Blatherwick and Long,[24] and others.

Sherman and Hawley [20] have recommended 750 to 1000 cc. of milk per day for the growing child. The calcium derived from this amount of milk, together with that obtained from other dietary sources, would provide about 1 gram of the element.

That growth is not necessarily a criterion of the adequacy of the calcium supply has been stressed by Sherman and Booher.[25] In experiments on rats they found that increase of body weight may be practically the same on rations containing from 0.16 to 0.5 per cent calcium. More calcium is stored on the higher levels of intake. Accordingly, Sherman and Booher have suggested that a calcium-poor condition of the body may coexist throughout much or all of the period of growth with normal increases of height and weight. It is considered, moreover, that the maximal rate of calcium retention is the optimal, until the body has attained the normal percentage of calcium for its age.

The calcium factor is of extraordinary importance during pregnancy and lactation. The demands of the fetus on the maternal organism are considerable. It has been estimated that the fetus at 40 weeks contains approximately 24 grams of calcium.[26] Under normal conditions of nutrition the maternal organism is in *positive* calcium balance and stores an amount of this element far in excess of the fetal requirement. So constant is this tendency in the human individual,[27] as well as in

[21] *Ibid.*, **35**, 353 (1918).

[22] The poor utilization of the calcium of spinach has been related to its presence therein as oxalate. See M. L. Fincke and H. C. Sherman, *J Biol. Chem.*, **110**, 421 (1935).

[23] *Ibid.*, **41**, 349 (1920).

[24] *Ibid.*, **52**, 125 (1922).

[25] *Ibid.*, **93**, 93 (1931); H. C. Sherman, *J. Am. Med. Assoc.*, **97**, 1425 (1931).

[26] M. H. Givens and I. G. Macy, *J. Biol. Chem.*, **102**, 7 (1933); I. G. Macy and H. A. Hunscher, *Am. J. Obst. Gyn.*, **27**, 878 (1933); L. McIlroy, *Proc. Roy. Soc. Med.*, **28**, 1385 (1935); V. Iob and W. W. Swanson, *Am. J. Diseases of Children*, **47**, 302 (1934).

[27] C. M. Coons and K. Blunt, *J. Biol. Chem.*, **86**, 1 (1930); I. G. Macy and coworkers, *ibid.*, **86**, 17 (1930).

experimental animals,[28] particularly toward the end of pregnancy, that it may be inferred that the storage of calcium is physiological, anticipating later emergencies and requirements. Retention of phosphorus accompanies that of calcium. During the last two months of pregnancy the daily calcium requirement is in excess of 2 grams. Climate, and particularly sunshine, is an important consideration. Coons [29] found that whereas a mean intake of only 1.4 grams of calcium and 1.6 grams of phosphorus was adequate for a group of pregnant women in Oklahoma, it was insufficient for a similar group in Chicago.

With the onset of lactation it is not uncommon to find *negative* calcium and phosphorus balances in spite of large intakes of these elements (Hunscher).[30] In time the balance is restored in the well-nourished individual, and later, when less milk is secreted, both calcium and phosphorus may be stored in the maternal body.[31] Better utilzation of calcium and phosphorus in the nursing mother may be obtained through the daily administration of cod-liver oil and yeast (Macy and associates).[32]

Cox and Imboden [33] determined the rôle of calcium and phosphorus in relation to reproductive success in rats. They employed as criteria of success the following: weight of the young at 21 days of age; ash content of the young at 21 days; and change of weight of the mother. It was found that success in reproduction and lactation were dependent on both the level of intake and the ratio of the mineral elements. The best results were obtained on a diet containing 0.49 per cent each of calcium and phosphorus, corresponding to a daily intake of approximately 42 mg. of each element. Reproductive success was minimal when the diet contained 2.45 per cent calcium and 0.245 per cent phosphorus.

In view of these circumstances, the effects of a low calcium intake during pregnancy and lactation when the needs of both the mother and child are involved may be readily imagined. Calcium and phosphorus deficiency under these conditions results in decalcification of the bones [34] and the loss of teeth. If the depletion of mineral from the bones is sufficient, they tend to become soft, a condition described as *osteomalacia*. The victims become badly deformed, owing to the flexibility of the bones. Osteomalacia is said to be very prevalent among women in certain parts of India and China. One of the cases described by Maxwell [35] was that of a Chinese woman, 7½ months pregnant, whose

[28] H. Goss and C. L. A. Schmidt, *ibid.*, **86**, 417 (1930).

[29] C. M. Coons and K. Blunt, *J. Biol. Chem.*, **86**, 1 (1930); Coons, *et al.*, *Oklahoma Agri. Exp. Sta. Bull.*, 223 (1935).

[30] *J. Biol. Chem.*, **86**, 37 (1930).

[31] For studies of the calcium balance of cows during lactation, see E. B. Forbes, *et al.*, *ibid.*, **52**, 281 (1922); E. B. Hart, *ibid.*, **54**, 75 (1922).

[32] *Ibid.*, **86**, 59 (1930).

[33] *J. Nutrition*, **11**, 147, 177 (1936).

[34] See for example K. U. Toverud and G. Toverud, *Biochem. J.*, **26**, 1424 (1932).

[35] J. P. Maxwell, "Osteomalacia and Diet," *Nutrition Abstracts & Revs.*, **4**, 1

calcium intake was only 0.17 gram per day and the phosphorus 0.35 gram. The serum calcium and phosphorus were 3.6 and 2.2 mg., respectively. The patient, on admission, was in a state of tetany.

Calcium deficiency is an important factor in the development of rickets, as will be seen shortly in another connection (p. 612). Other phases of calcium metabolism are reserved for the discussion on bone (p. 625).

The following foods are among the better sources of calcium: cheese, milk, egg yolk, beans, lentils, wheat bran, cottonseed meal, linseed meal, nuts, dried figs, leafy vegetables such as cabbage, especially the outermost leaves, turnip tops, cauliflower, asparagus. The animal organism is also capable of utilizing inorganic and organic salts of calcium, such as the carbonate, phosphate, and lactate.[36]

The Phosphorus Requirement. The intimate relation between calcium and phosphorus metabolism has been referred to in the preceding paragraphs. Both enter into the structure of bone, and either may in consequence be the limiting factor in growth. Moreover, large amounts of phosphorus are utilized for other purposes. Birds require this element, as well as calcium, in the production of eggs. In the lactating mammal, phosphorus is needed in the formation of casein and other milk constituents.

The requirement of the growing animal for phosphorus is relatively high. This is also true for the pregnant or lactating individual. In the adult, where phosphorus is required merely to replace the loss from the body, the minimum requirement averages about 0.88 gram per day. In his statistical studies, Sherman found that only 4 per cent of the American dietaries examined fell below this level. As a rule, there is therefore for the adult less danger of phosphorus deficiency than of calcium deficiency. Phosphorus deficiency is also an important factor in the nutrition of cattle, particularly in regions where the soil and vegetation are poor in this element. Cattle may develop an intense craving for phosphorus, which manifests itself in bone-eating. This condition is known as osteophagia. An outbreak of this abnormality in South Africa has been described by Green,[37] who states that the craving may be produced experimentally upon phosphorus-low rations and removed by administration of phosphorus compounds and by phosphatic manuring of the soil.

The condition of *aphosphorosis* (phosphorus deficiency) in ruminants and the condition of *acalcerosis* (calcium deficiency) which may accompany it are evidently much more prevalent and widely distributed than

(1934); the reader is also urged to consult R. C. Garry and D. Stiven, *ibid.*, **5**, 855 (1936).

[36] T. B. Osborne and L. B. Mendel, *J. Biol. Chem.*, **34**, 131 (1918); see also H. Steenbock and co-workers, *ibid.*; **56**, 375 (1923).

[37] *Ibid.*, **46**, Proc. xix (1921); *Physiol. Rev.*, **5**, 336 (1925).

was formerly appreciated. An admirable survey of the subject has recently been prepared by Sir Arnold Theiler and Green.[38]

Among the richer sources of phosphorus are the following foods: cheese, milk, egg yolk, beans, peas, lentils, bread, especially rye and whole wheat, fish, meat, cottonseed meal, linseed meal, oatmeal, barley. The phosphorus requirement may be supplied, even in the growing animal, exclusively from inorganic sources (Osborne and Mendel).[36]

As will be seen presently, phosphorus deficiency is an important factor in rickets. It is now also recognized that a proper balance must exist between the intake of calcium and phosphorus. The most favorable Ca : P ratio for growth and bone formation is evidently between 1 and 2. Even if the absolute amount of phosphorus is unchanged a rise in the Ca : P ratio to 5 is associated with a pronounced decrease in growth, bone ash content, and the percentage of inorganic phosphorus in the blood serum (Bethke, Kick, and Wilder).[39] As we have seen, the Ca : P ratio is also of importance in determining reproductive success (Cox and Imboden[33]). In the pregnant and lactating rat, optimum results were obtained when the ratios were 1 : 1 and 1.5 : 1.

Experience in the rearing of lions and other carnivorous animals in several zoological gardens has brought out very strikingly the importance of calcium and phosphorus in nutrition. It is now evident that the difficulty formerly encountered in raising animals in captivity was due largely to the fact that the diet, after weaning, was inadequate, consisting as it did almost entirely of raw meat. Even lions, when they are young, find it difficult to chew large bones, and this was formerly the chief source of calcium and phosphorus provided to them. As a result, the young animals kept on this diet frequently developed a severe form of rickets and succumbed. However, when the diet was supplemented by the addition of calcium- and phosphorus-rich food, such as milk and crushed bones, and by the addition of cod-liver oil (the latter contains the antirachitic vitamin), the animals grew normally and the condition of those which had previously developed rickets was frequently improved.

Other aspects of phosphorus metabolism will be considered in the section on bone (p. 612).

Magnesium. It is usually taken for granted that magnesium deficiency occurs very rarely, if at all, except in the experimental animal restricted to a diet poor in magnesium. The relative abundance of this element in most plant and animal tissues, coupled with certain observations which indicate that the normal requirement is low, has made this conclusion a plausible one. However, there is the possibility that magnesium deficiency may occur, but that it has been overlooked, since it is only recently that experimental studies have disclosed the nature of the

[38] *Nutr. Abst. Rev.*, **1**, 359 (1931–32).

[39] *J. Biol. Chem.*, **98**, 389 (1932); see also Brown, Shohl, *et al.*, *ibid.*, **98**, 207 (1932).

symptoms and of the pathological changes associated with this condition.

Of outstanding importance in this connection has been the work of McCollum, Kruse, and their associates,[40] which has not only added to our knowledge of the requirement of magnesium for growth and other bodily activities, but which has also established the fact that it is essential to life. A diet containing only 1.8 parts per million of magnesium was fed to young rats. Within 3 to 5 days the exposed skin areas of these animals became vividly red from vasodilation and hyperemia. Soon hyperirritability of the nervous system developed, followed by cardiac arrhythmia, spasticity, and tonic-clonic convulsions. On the same diet in dogs the symptoms of vasodilation, hyperexcitability, and tetany were less intense, but the trophic and nutritive changes were more conspicuous than in the rat. The tetany is described as distinct from other forms, being characterized by vasomotor spasm and the absence of both carpopedal spasm and laryngospasm.

Magnesium deficiency results in a progressive decrease of the serum magnesium content, a rise in cholesterol, chiefly in the form of esters, and a commensurate decrease in total fatty acids. Kruse and associates have related the low serum magnesium to the development of tetany, while the changes in the blood lipids have been interpreted as a reflection of failing fat metabolism.

It seems that, in the early stages of magnesium deficiency, conservation of calcium in the bones is promoted; the weight of the bone increases, as does the total ash content, including calcium and phosphorus. As the deficiency continues, the tendency to conserve calcium is reversed. It has been determined that the effects of combined calcium and magnesium deprivation resemble more nearly the effects of calcium deficiency alone. It is of interest that the symptons of magnesium tetany which develop as serum magnesium is reduced fail to appear if there is a simultaneous reduction of serum calcium.

Magnesium deficiency in the rat has been associated with the development of degenerative changes in the heart and especially in the kidneys.[41]

Milk is a relatively poor source of magnesium; the concentration in cows' milk is about 0.01 per cent. A diet consisting of milk and supplemented with adequate amounts of cod-liver oil, yeast, and such minerals as Fe, Cu, Mn, when fed to calves leads to a lowering of the magnesium content of the blood. These animals eventually develop tetany and die, even though the calcium and phosphorus are normal (Duncan, Huffman, and Robinson [42]).

[40] H. D. Kruse, E. R. Orent, and E. V. McCollum, *J. Biol. Chem.*, **96**, 519 (1932); **100**, 603 (1933); Orent, Kruse, and McCollum, *Am. J. Physiol.*, **101**, 454 (1932); *J. Biol. Chem.*, **106**, 573 (1934); Kruse, M. M. Schmidt and McCollum, *ibid.*, **106**, 553 (1934); H. G. Day, Kruse, and McCollum, *ibid.*, **112**, 337 (1935).

[41] D. M. Greenberg, C. E. Anderson, and E. V. Tufts, *J. Biol. Chem.*, **114**, xliii (1936); Tufts and Greenberg, *ibid.*, **122**, 693, 715 (1938).

[42] *Ibid.*, **108**, 35 (1935).

Judging from the observations of Hirschfelder,[43] the occurrence of magnesium deficiency clinically is not outside the range of possibility. In ten patients kept on a special soft diet which included large amounts of milk he found the plasma magnesium to be markedly low. This was associated with hyperirritability of the neuromuscular system, muscular twitchings, and convulsions.

On the basis of a study of the magnesium balance in a group of children (4 to 7 years old), Daniels and Everson[44] estimated the daily requirement of magnesium to be not less than 13 mg. per kilogram of body weight.

Of interest are the earlier observations of Mendel and Benedict,[45] who noted that, in dogs, cats, and rabbits, an increased excretion of magnesium could be induced by the administration of calcium, and that an increased elimination of calcium could be brought about by the administration of magnesium. Similar relations have been shown to hold for man by Bogert and McKittrick.[46]

Approximately two-thirds of the magnesium in the body is present in bone. The analyses of Hammett[47] show that the ash of the femur and humerus of rats contains slightly less than 1 per cent magnesium and that this value decreases somewhat with age. More magnesium than calcium is present in muscle tissue. Katz[48] analyzed fresh human muscle and found it to contain 0.212 part per thousand of magnesium as compared with 0.075 part per thousand of calcium.

Medes[49] found little variation in the concentration of magnesium in rats. Analyses of a series of whole animals, at 29, 60, and 90 days of age, revealed the interesting fact that the magnesium content remained constant during growth, the amount determined in all cases being 0.045 per cent. These observations are similar to those of Buckner and Peter,[50] who had shown previously that, whereas the percentages of phosphorus and calcium increased with age, the magnesium content remained about the same percentage of the body weight. It is of interest to note that the contents of calcium, potassium, and magnesium are higher in female than in male rats.

Iron. Sherman[51] placed the iron requirement of the adult at approximately 10 mg. per day. He expressed the opinion that there is comparatively little danger of iron deficiency in freely chosen diets. A similar amount of iron is sufficient to meet the maintenance and growth

[43] *J. Am. Med. Assoc.*, **102**, 1138 (1934).
[44] *J. Nutrition*, **11**, 327 (1936).
[45] *Am. J. Physiol.*, **25**, 1, 23 (1909–10).
[46] *J. Biol. Chem.*, **54**, 363 (1922).
[47] *Ibid.*, **64**, 693 (1925).
[48] *Arch. ges. Physiol.*, **63**, 1 (1896).
[49] *J. Biol. Chem.*, **68**, 295 (1926).
[50] *Ibid.*, **54**, 5 (1922).
[51] *Harvey Lectures* (1917–19), p. 117; see also G. E. Farrar and S. M. Goldhamer, *J. Nutrition*, **10**, 241 (1935).

requirements of children, according to recent investigations.[52] In a study of the iron balance in women during pregnancy, Coons [53] found that, with intakes varying from 9.69 to 19.45 mg. per day, the retention varied from +0.88 to +6.97 mg. The iron was in negative balance only once in twenty-three determinations (−2.2 mg.). It is thus seen that the maternal organism assimilates during the period of pregnancy enough iron to supply the new-born infant the needed reserves. Unfortunately, the diet of the gravid woman is often inadequate in respect to iron, which accounts in part for the frequency of anemia during pregnancy.[54]

Most animals are born with an extra supply of iron (the guinea pig is an exception) which is utilized during the early period of life when milk is the chief, if not the sole, article of diet. Milk is very low in iron. Human milk contains from 1 to 2 mg. per liter. Even lower values have been recorded for cows' milk. If an animal's diet is restricted to milk for much longer than its normal lactation period, anemia may result, as has been shown by Bunge,[55] Abderhalden,[56] and numerous other workers.

Considerable interest has been aroused in the problem of nutritional or milk anemia since the discovery by Hart that copper is an effective agent in stimulating the utilization of iron and the production of hemoglobin and of red blood corpuscles. If sufficiently purified, iron fails to correct this form of anemia in rats and rabbits, according to Hart [57] and other observers.

One of the richest sources of iron is to be found in organ meats, or "extra carcass parts" (liver, heart, kidney, spleen). In addition to meat, other good sources among foods are: egg yolk, whole wheat, fish, oysters, clams, nuts, dates, figs, beans, lentils, asparagus, spinach, molasses, oatmeal. Foods differ in respect to the availability of the iron which they contain. Whereas more than half of the iron present in meat, liver, wheat, and oats is utilizable, less than one-quarter of that contained in spinach is available for physiological use. The iron of egg yolk is also partly unavailable for hemoglobin formation. It has been found that it takes twice as much iron in the form of liver as of whole wheat to get an equivalent amount of blood regeneration.[58]

Iron salts, such as ferric chloride or pyrophosphate can function as the sole source of iron for hemoglobin synthesis.[59]

[52] M. S. Rose, *et al.*, *J. Nutrition*, **3**, 229 (1930–31); J. M. Leichsenring and I. H. Flor, *ibid.*, **5**, 141 (1932); see also A. L. Daniels and O. Wright, *ibid.*, **8**, 125 (1934).

[53] *J. Biol. Chem.*, **97**, 215 (1932).

[54] Anemia due to deficient diet is a common occurrence both in animals (especially hogs) and in the human population. It is a cause of poor growth and increased susceptibility to infection in infants and adult women (L. S. P. Davidson and I. Leitch, *Nutrition Abstracts & Revs.*, **3**, 901 (1934).

[55] *Z. physiol. Chem.*, **13**, 399 (1889).

[56] *Z. Biol.*, **39**, 193 (1900).

[57] Hart, Steenbock, Elvehjem, and Waddell, *J. Biol. Chem.*, **77**, 797 (1928); **83**, 243, 251 (1929); **84**, 115 (1929).

[58] M. S. Rose and E. McC. Vahlteich, *ibid.*, **96**, 593 (1932); Rose, Vahlteich, and G. Macleod, *ibid.*, **104**, 217 (1934).

[59] C. A. Elvehjem, E. B. Hart, and W. C. Sherman, *ibid.*, **103**, 61 (1933).

Steenbock [60] found that females, as they attain maturity, store more iron than males. Since the injection of ovarian extract into immature rats promotes storage, whereas castration exerts the opposite effect, it has been surmized that the sexual differentiation in iron metabolism is probably related to the function of the ovaries.

Copper. This element is probably present in all plant and animal tissues. Analysis of a large variety of foods has shown that none is entirely devoid of copper, although there is a wide range of variation. For example, fresh celery contains only 0.1 mg. of copper per kilogram, whereas fresh calf liver often contains more than 40 mg. per kilogram. Milk is relatively poor, containing approximately 0.6 mg. per liter (0.6–0.7 for cows' milk according to Hess, Supplee, and Bellis; [61] 0.4 to 0.6 mg. for human milk).

Outside of its special rôle as a component of the hemocyanin molecule and its occurrence in the pigment turacin (p. 248), copper seems to play, in conjunction with iron, an important biological function in the production of hemoglobin. This was first demonstrated by Hart and co-workers [57] in the type of nutritional anemia produced in rats restricted to a milk diet. Purified iron alone is ineffective. Certain investigators have contended, however, that iron alone is an effective hematopoietic agent; others have reported that nickel, germanium, manganese, etc., produce the same effect as copper. However, the weight of evidence at present is that the effect of copper in hemoglobin formation is specific and that other elements do not have this stimulating effect.[62]

Elvehjem and Sherman [63] have attempted to determine more precisely the function of copper in hematopoiesis. They found that the addition of pure iron to the milk fed to anemic rats increased the total iron content of the liver and spleen. However, no increase in hemoglobin was observed. When copper was substituted, the stored iron in the liver was used directly in the building of hemoglobin. As compared with the decreased iron content in the liver, the change in the spleen was slight.

In a more recent study, Schultze, Elvehjem and Hart [64] found that deficiency of iron alone may produce anemia in young pigs. If the bodily stores of copper are preserved the anemia responds to treatment with iron only. However, if there is also a coexisting deficiency of copper, hemoglobin and erythrocytes are not formed when pure iron alone is supplied.

Oysters from certain localities have been found to exert a marked beneficial effect in milk anemia, presumably because of their high copper

[60] *Ibid.*, **114**, ci (1936).
[61] *Ibid.*, **57**, 725 (1923).
[62] For fuller details and bibliography, the reader is referred to C. A. Elvehjem, *Physiol. Rev.*, **15**, 471 (1935).
[63] *J. Biol. Chem.*, **98**, 309 (1932); see also H. W. Josephs, *ibid.*, **96**, 559 (1932).
[64] *Ibid.*, **116**, 93 (1936).

content.[65] Robscheit-Robbins, Elden, Sperry, and Whipple[66] have observed that apricots, which are high in copper, are likewise effective in stimulating blood regeneration. Among other abundant sources of copper are the following: liver (calf liver more than beef liver), cocoa, molasses, nuts, currants, split peas.

From balance experiments on three adult subjects, Chou and Adolph[67] have determined that the copper requirement of man is approximately 2 mg. per day. It is estimated that the adult body contains a total of 100 to 150 mg. of copper.

That copper may accumulate in tissues owing to its presence in foodstuffs is to be expected. Yet it is a curious fact that fetal and infant organs (brain,[68] liver[69]) contain a greater amount of copper than is present in adult tissues, which argues against the purely adventitious origin of copper in the organism.

Iodine. The indispensability of iodine is firmly established. It is utilized partly in the production of thyroxine, the hormone of the thyroid gland and may have other functions that are as yet unknown. Considerable amounts of iodine occur in the ovary. In regions where the water, soil, and in consequence the vegetation are iodine-poor, goiter is prevalent.[70] On the other hand, where iodine is abundant goiter is rare. Among the Japanese, for example, goiter is practically non-existent, there being about one goiter per million of population. It is instructive to note that the iodine intake of the Japanese is probably greater than that of any other people, owing to the fact that seaweed is a common ingredient of the diet. Seaweed contains about 1000 times as much iodine as any other food (McClendon).[71]

Lunde and Closs[72] have estimated that the amount of iodine necessary to meet all the requirements of metabolism is about 0.05 mg. per day. According to Fellenberg,[73] a third of this amount is sufficient for the maintenance of iodine balance in an adult. Endemic goiter could probably be prevented in many instances by about 50 mg. of iodine a year administered at proper intervals. Larger amounts (400 mg. of potassium iodide a year) are, however, recommended. During pregnancy and lactation, at puberty and the menopause, as well as during

[65] H. Levine, R. E. Remington, and F. B. Culp, *J. Nutrition*, **4**, 465 (1931).
[66] *J. Biol. Chem.*, **79**, 563, 577 (1928).
[67] *Biochem. J.*, **29**, 476 (1935).
[68] M. Bodansky, *J. Biol. Chem.*, **48**, 361 (1921).
[69] D. B. Morrison and T. P. Nash, *ibid.*, **88**, 479 (1930).
[70] For the geographical distribution of goiter, as well as for the historical aspects of the subject, the reader is referred to C. R. Harington, "The Thyroid Gland," London, 1933, Chap. III.
[71] *J. Biol. Chem.*, **102**, 91 (1933).
[72] *Nord. Med. Tidskrift.*, **1**, 475 (1929); cited by W. Weston, *Am. J. Pub. Health*, **21**, 715 (1931).
[73] *Biochem. Z.*, **142**, 246 (1923); see also L. Scheffer, *ibid.*, **259**, 11 (1933); *Klin. Wochschr.*, **12**, 1285 (1933); V. Cole and G. M. Curtis, *J. Nutrition*, **10**, 493 (1935).

infections, the iodine requirement is believed to be somewhat above normal.

Sea foods constitute a rich source of iodine. Fish contain about 1 mg. per kg. The quantity is variable, however, depending on the locality and the food supply. Oysters have been found to contain an even larger amount, 16 mg. per kg. South Carolina spinach has been found to contain 800 to 1200 parts of iodine per billion, on a dry basis, whereas, according to the analyses of McClendon and Remington,[74] California spinach contained only 32 parts per billion. Similar differences, though not of the same magnitude, have been noted for other vegetables—beans, asparagus, potatoes, lettuce, carrots, etc. Data on the iodine content of hospital foods have been recently published by Cole, Curtis, and Bone.[75]

Other Mineral Constituents. When evidence first began to accumulate that certain elements, such as copper, zinc, manganese, nickel, and cobalt, were present in traces in a large variety of plant and animal tissues, no particular importance was attached to the findings. It seemed logical to suppose that their occurrence was essentially adventitious. Gradually, however, the point of view has changed and increasing effort has been directed in the attempt to evaluate the nutritional and physiological significance of the so-called "trace" elements.[76]

Zinc. Hubbell and Mendel[77] observed a slight retardation in the growth of mice fed a highly purified diet that was practically free from zinc (0.005 mg. was the estimated daily intake on this diet). When the total zinc intake was increased to 0.02 mg., growth was more nearly normal; larger amounts retarded growth. The importance of zinc in nutrition has been confirmed in Hart's laboratory,[78] where it was found that on a low-zinc diet (1.6 mg. per kg. of food) the growth of rats was markedly retarded; there was in addition faulty development of the fur and the rats seemed to be abnormally active and hyperirritable. These results, as well as others obtained in Bertrand's laboratory[79] and elsewhere, strongly support the view that zinc is an indispensable ele-

[74] *J. Am. Chem. Soc.*, **51**, 394 (1929).

[75] *J. Am. Dietetic Assoc.*, **10**, 200 (1934).

[76] For a general review of the nutritional significance of the mineral elements occurring in traces in the animal body, the student is referred to M. S. Rose, *Yale J. Biol. Med.*, **4**, 499 (1932); see also "Report of the Committee on Growth and Development, White House Conference on Child Health and Protection," Part III, pp. 282–306, Century Co., 1932. The recent literature has been summarized by E. B. Hart and C. A. Elvehjem, *Ann. Rev. Biochem.*, **5**, 271 (1936). The rôle of boron, copper, zinc, manganese, etc., in plant nutrition has been reviewed by P. Mozé, *ibid.*, p. 525 (1936).

[77] *J. Biol. Chem.*, **75**, 567 (1927).

[78] W. R. Todd, C. A. Elvehjem, and E. B. Hart, *Am. J. Physiol.*, **107**, 146 (1934); F. E. Stirn, C. A. Elvehjem, and E. B. Hart, *J. Biol. Chem.*, **109**, 347 (1935).

[79] G. Bertrand and R. C. Bhattacherjee, *Compt. rend.*, **198**, 1823 (1934); *Bull. soc. sci. hyg. aliment.*, **23**, 369 (1935).

ment in nutrition;[80] however, owing to its widespread distribution in foods, it is unlikely that zinc deficiency ever occurs in animals, except when induced experimentally. That it may occur naturally in plant life has been reported by Finch and Kinnison.[81]

What the rôle of zinc may be in animal nutrition is essentially unknown, but certain observations suggest a connection with insulin and possibly with other hormones. Insulin, even in purest crystalline form, contains zinc, which is present apparently in firm combination in the molecule.

Zinc occurs in practically all tissues but is most abundant in liver and pancreas; cows' milk contains approximately 2.5 mg. per liter.

Manganese. Disturbance of the estrus cycle has been observed by Kemmerer, Elvehjem, and Hart[82] in mice kept on a manganese-deficient diet. Although this effect was not evident in rats in the experiments of Orent and McCollum,[83] there was, however, failure in the development of the mammary tissue in the females with the result that the mothers were incapable of suckling their young. In male rats complete degeneration of the germinal epithelium occurred. These effects were avoided when the manganese-free rations were supplemented by the addition of small amounts of manganese (0.005 to 0.05 per cent). Results differing from those of Orent and McCollum have been obtained by Daniels and Everson,[84] who found that failure of the young to suckle was due to their own weakness rather than to deficient mammary function. Mothers on a manganese-low diet were able to rear the young taken from mothers kept on a normal diet; it therefore seemed that manganese deficiency exerted a deleterious effect on the development of the fetuses.

The analyses of Richards,[85] Peterson and Skinner,[86] and others have established the widespread occurrence of manganese in plant and animal tissues.[87] This element is especially abundant in blueberries and lettuce and seems to be a constant constituent of the reproductive organs of plants and animals. Even though the evidence is not yet complete, there is much to suggest that it may be essential for growth and more specifically for normal reproduction both in plants and animals. Balance experiments on children conducted by Everson and Daniels[88] places the daily requirement at 0.2 to 0.3 mg. per kg. of body weight.

[80] See, however, J. M. Newell, and E. V. McCollum, *J. Nutrition*, **6**, 289 (1933).
[81] *Univ. Ariz. Agr. Exp. Sta., Tech. Bull.*, **47**, (1933).
[82] *J. Biol. Chem.*, **92**, 623 (1931).
[83] *Ibid.*, **92**, 651 (1931).
[84] *J. Nutrition*, **9**, 191 (1935).
[85] *Biochem. J.*, **24**, 1572 (1930).
[86] *J. Nutrition*, **4**, 419 (1931).
[87] The literature on the distribution of manganese has been reviewed by F. von Oettingen, *Physiol. Rev.*, **15**, 175 (1935).
[88] *J. Nutrition*, **8**, 497 (1934).

Aluminum. The question of the nutritional requirements of aluminum has received very little attention and consequently its significance from this standpoint is undetermined. Observations in McCollum's laboratory[89] indicate that it is probably non-essential. Aluminum is the most abundant metallic element in the earth's crust and has been found in a large variety of plant and animal tissues.[90] Partly because of the widespread use of aluminum cooking utensils, the question of its toxicity has been intensively studied. Although the problem can in no sense be considered closed, there is at present no evidence that aluminum, in amounts even considerably larger than those ordinarily encountered, is toxic for man.

Nickel and Cobalt. There is at present no evidence either that these elements are essential or that they are non-essential in nutrition. The interesting observation has been made in R. C. Lewis' laboratory [91] that cobalt produces a characteristic polycythemia in young rats.

Fluorine. Sharpless and McCollum have shown[92] that rats grow normally on a diet low in fluorine and that the content of this element in bones and teeth can be reduced to a negligible amount (6–25 parts per million in bone; practically 0 in teeth) without any gross evidence of deleterious effect. This disposes of earlier assumptions of the essential nature of fluorine in bone formation. Certain it is that, if it is in any sense required by the organism, the amount necessary is extremely small. On the other hand, it has now been established that fluorides are toxic, interfering particularly with the retention and deposition of calcium in teeth and bones. Mottled teeth, a condition in which the enamel deteriorates, owing to the absence of intercementing material, are very prevalent in certain sections of the United States and other parts of the world. This abnormality has been recently associated with the presence in the drinking water of excessive amounts of fluoride.[93] In one community in which mottled enamel is endemic, the drinking waters were found to contain 3.8 to 7.15 mg. per liter of fluorine, as compared with 0 to 0.3 mg. in waters of other localities where this condition is not endemic (Smith, Lantz, and Smith).[94] It was found that every child exposed to the environmental conditions of this community during the years of growth of the enamel of the permanent teeth was certain to have mottled enamel.[95]

[89] E. V. McCollum, O. S. Rask, and J. E. Becker, *J. Biol. Chem.*, **77**, 753 (1928).

[90] V. C. Myers and J. W. Mull, *J. Biol. Chem.*, **78**, 625 (1928); Myers and D. B. Morrison, *ibid.*, **78**, 615 (1928); F. P. Underhill, F. I. Peterman, *et al.*, *Am. J. Physiol.*, **90**, 1–82 (1929).

[91] *J. Biol. Chem.*, **96**, 11 (1932).

[92] *J. Nutrition*, **6**, 163 (1933).

[93] F. S. McKay, *J. Dental Research*, **10**, 561 (1930).

[94] *Ariz. Agr. Expt. Sta. Tech. Bull.* 32, June, 1931.

[95] The increasing interest in the problem of the physiological effects of fluorine is evidenced by the recent appearance of two comprehensive reviews on the subject: F. J. McClure, *Physiol. Rev.* **13**, 277 (1933); F. DeEds, *Medicine*, **12**, 1 (1933).

Schour and Smith are of the opinion that fluorine exerts a direct local action on

Bromine, silicon, arsenic, and boron are known to occur in many tissues, but their physiological significance in the animal organism has not been determined. A considerable amount of work has been done in recent years on *selenium*. This element is present in the soil in varying amounts; in certain localities, the vegetation (wheat, etc.) contains sufficient quantities to be toxic when fed to animals (cattle, swine, etc.). The eggs and milk in these areas are said to contain selenium. Selenium seems to be an inhibitor of biological oxidations; in animals it produces a wide variety of symptoms.[96]

THE PROTEIN REQUIREMENT IN NUTRITION AND THE RÔLE OF THE INDIVIDUAL AMINO ACIDS

The subject which we are about to consider has had a remarkable and steadily progressive development. Originally interest was confined largely to the question of what constituted the minimum requirement of protein for nutrition. Then came the realization that all proteins were not alike in their nutritive value, that there were qualitative and quantitative differences which determined their biological usefulness. Soon thereafter it was disclosed that these differences in the nutritional value of various proteins were related to their amino-acid composition; that the inadequacy of certain proteins for the purpose of growth and other bodily functions was due to a deficiency with respect to one or more amino acids, such as lysine or tryptophane. At the time these discoveries were being made, there were known to be about eighteen amino acids; it was therefore logical for students of nutrition to pursue the problem further with the object of ascertaining which of these were essential to nutrition and which were not. It was assumed that those amino acids which the organism could synthesize were dispensable, whereas those which it could not synthesize had to be provided in the diet and were therefore indispensable.

In the earlier investigations the procedure consisted in preparing mixtures of amino acids by digesting protein. From such digests certain amino acids could be removed, e.g., histidine, arginine, tyrosine, or tryptophane. If the residual mixture proved nutritionally inadequate, and if by adding the missing amino acid the deficiency was corrected, it was considered as evidence that the added amino acid was an essential one. Although important knowledge was gained in this way, it soon became obvious that further progress would depend on the use, not of digests, the composition of which was known only approximately, but of known mixtures of highly purified amino acids. This idea was embodied

the enamel-forming cells and that the changes observed in the enamel and dentine are not produced primarily by changes in blood calcium and phosphorus or by disturbance in parathyroid function (*J. Am. Dental Assoc.*, **22**, 796 [1935]).

[96] For fuller details and references to the literature the reader should consult Hart and Elvehjem, *Ann. Rev. Biochem.*, **5**, 271 (1936).

in the work of Rose [97] and his students who discovered that a mixture of the known amino acids, when fed to rats as the sole source of nitrogen, failed to support growth. As we shall see, this led to the isolation and identification of a new indispensable amino acid, α-amino-β-hydroxybutyric acid. By including this new essential, Rose finally succeeded in rearing rats on a ration in which the protein component was entirely replaced by a known mixture of highly purified amino acids. With this development the way was opened to a clear-cut differentiation of the various amino acids with regard to their importance in nutrition and to the solution of numerous other problems.

Amount of Protein Needed. We may now return to the topic which formerly absorbed the interests of many students of nutrition, namely that of the protein requirement. This was at one time estimated from statistical data. For example, Voit found that for adults in Germany the average daily consumption of protein was 118 grams. Atwater in this country, and other workers both in America and in Europe, made similar estimates of the average protein intake. This quantity, therefore, was formerly accepted by many students of nutrition as representing an adequate supply. As this amount of protein provides less than 500 calories, the energy needs of the body must obviously be met largely from carbohydrate and fat. From this standpoint, a well-balanced diet for an individual engaged in moderate physical work may include 50–60 grams of fat (465 to 560 calories) and about 500 grams of carbohydrate (approximately 2000 carlories).

Chittenden [98] studied the protein requirement in human nutrition very exhaustively and reached the conclusion that the Voit standard of 118 grams was far in excess of the actual needs of the body. In his investigations were included persons engaged in various occupations (soldiers, professors, students, athletes, etc.). Chittenden determined that the nitrogen requirement per day per kilogram of body weight was fairly uniform for different individuals and amounted to 0.10 to 0.12 gram. A man weighing 70 kg. would therefore require 7–8.4 grams daily, or 44–53 grams of protein. Accordingly, an allowance of 60 grams of protein per day should be entirely adequate. This calls for two assumptions, namely, that there is adequate provision, through other food elements, to meet the energy requirements, and that the protein ingested provides a complete and adequate assortment of all the amino acids essential to the formation of tissue protein. These assumptions cannot always be made, for not all proteins are of equivalent nutritive value. It is evident that fixed standards are of limited worth. What the optimum proportions of the various foodstuffs will be in any particular case will depend on many circumstances. Thus, in cold climates a high level of protein intake is dictated by sound physiological

[97] *Science*, **86**, 298 (1937). See also p. 568 of this book.

[98] R. H. Chittenden, "Physiological Economy in Nutrition," F. A. Stokes, New York, 1904.

reasoning. In warm climates a lower protein level would seem more suitable.[99] Fortunately, in some particulars, the dietary habits of people frequently tend in the proper direction.[100]

The absolute minimum evidently falls considerably below the standard set by Chittenden. Numerous investigators have attempted to determine the minimum protein intake sufficient to maintain nitrogen balance. Folin [101] obtained a minimum excretion of 2.6 grams of nitrogen on the twelfth day of an experiment on a low-protein diet. The subject of this experiment weighed 64.0 kg. In a similar experiment Thomas [102] (body weight 76.2 kg.) obtained a minimum excretion of 2.98 grams on the nineteenth day. Deuel [103] was able to reduce his nitrogen elimination to a minimum of 2.1 grams. The injection of thyroxine was followed after an interval of 7 days by an increased output of nitrogen, due obviously to an increased "wear and tear" of the tissues. Smith,[104] by insuring for himself an abundant caloric supply in the form of carbohydrate and fat, was able to reduce his endogenous protein metabolism to an extremely low level, the lowest recorded in the literature. During the course of the last 24 days of a 28-day experiment, he excreted but 80.8 grams of nitrogen, or an average of 3.34 grams per day. The lowest point was reached on the twenty-fourth day of the experiment when the total nitrogen excreted in the urine was only 1.58 grams. These figures represent an amount of protein metabolism of 20 grams or less. From this discussion, the inference is not to be drawn, however, that a low protein intake is at all desirable. It is true that if the protein fed were one containing all the essential amino acids in suitable proportions for tissue synthesis, a daily allowance of 50 or 60 grams would be adequate. Proteins of animal origin are usually complete or adequate in this sense, but an adequate supply of the required amino acids can hardly be expected from 50 or 60 grams of many of the vegetable proteins, such as zein of corn, gliadin of wheat, hordein of barley,

[99] Denis and Borgstrom (*J. Biol. Chem.*, **61**, 109 [1924]) in a large group of students in a Southern medical school found an average daily urinary excretion of 10.63 grams of nitrogen. This figure, plus 10 per cent added to account for the nitrogen lost through the feces, indicates an average consumption of 73.8 grams of protein, an amount not much higher than Chittenden's standard and distinctly below the average protein intake (121 grams) recorded for inhabitants of the United States. During the winter months the same group of students showed higher values for nitrogen excretion than in April or July. Youngburg and Finch (*ibid.*, **68**, 335 [1926]) observed essentially the same level of protein intake in a group of medical students in the North and were unable to demonstrate seasonal variations in nitrogen excretion. Similar results reported by others show that the protein intake of students in the North and South, per 70 kg. body weight, is approximately the same (Brooks, *Am. J. Physiol.*, **89**, 403 [1929]); Beard, *ibid.*, **82**, 577 [1927]).

[100] See for example the entertaining article by L. B. Mendel, "The Changing Diet of the American People," *J. Am. Med. Assoc.*, **99**, 117 (1932).

[101] *Am. J. Physiol.*, **13**, 66 (1905).

[102] *Arch. Anat. Physiol.*, Physiol. Abt., 219 (1909); cited by Smith.

[103] Deuel, Sandiford, Sandiford, and Boothby, *J. Biol. Chem.*, **76**, 391 (1928).

[104] *Ibid.*, **68**, 15 (1926).

and phaseolin of kidney beans. It is therefore of the utmost importance to allow a liberal margin of safety, particularly when part of the protein is derived from plant sources.

Relation of Protein Intake to Kidney Disease. Whether a high protein intake is conducive to kidney damage and vascular disease has been a subject of much speculation and some clinical and experimental study. In the rat, feeding very large amounts of protein (70 to 90 per cent of the total food) produces renal hypertrophy, which may be related to the increased activity of the kidneys incidental to the excretion of excessive quantities of the end-products of protein metabolism. This effect may be exaggerated by removing one kidney, thereby throwing the entire burden of excretion on the remaining organ. Aside from the hypertrophy and some tubular change in the older animals, the kidneys remain as a rule essentially normal.[105]

Those [106] who have studied the dietary habits of Eskimos, whose daily consumption of protein may exceed 500 grams, have not observed any unusual prevalence of cardiac or renal disease among them. Nor has a detailed metabolic study of two distinguished Arctic explorers, maintained on an exclusive meat diet for one year, given any evidence suggesting that renal damage had occurred.[107] This evidence, however, is not conclusive proof that protein intake is entirely unrelated to kidney disease. It is possible that tolerance to protein may be a matter of adaptation, particularly in the Eskimo. In summarizing the information available at present, it may be said that the level of protein intake seems to have little to do with the spontaneous kidney and blood-vessel changes observed in lower animals and with cardiovascular-renal diseases found in man (Bischoff [108]).

Qualitative Differences in the Nutritional Value of Proteins. That the value of a protein in nutrition depends largely on the nature and amounts of the amino acids which it yields on hydrolysis seems to be definitely established. Not only are there differences in the proportions in which the amino acids are present in different proteins, but one or more amino acids are totally lacking in some of them. The question therefore arises as to whether a protein that does not have a complete assortment of amino acids can be adequate in nutrition when it is the sole protein provided in the diet. Much depends on the nature of the deficiency. Many proteins lack glycine, but this is not a limiting factor in nutrition because the animal organism is capable of synthesizing this amino acid from other substances that are available. On the contrary,

[105] T. S. Moise and A. H. Smith, *J. Exp. Med.*, **46**, 27 (1927).
[106] W. A. Thomas, *J. Am. Med. Assoc.*, **88**, 1559 (1927).
[107] E. Tolstoi, *J. Biol. Chem.*, **83**, 753 (1929).
[108] *J. Nutrition*, **5**, 431 (1932).
Slonaker has studied the influence of different levels of protein intake (10, 14, 18, 22, and 26 per cent) in the rat on growth, activity, fertility, lactation, mortality of young, duration of life, and other factors. *Am. J. Physiol.*, **96**, 547, 557; **97**, 15, 322, 573, 626; **98**, 266 (1931); **113**, 159 (1935).

a deficiency of tryptophane, leucine, histidine, lysine, or certain other amino acids, materially reduces the biological value of a protein. Maintenance and growth do not occur in the absence of any one of these, for their synthesis in the animal body does not take place.[109]

One method of studying the nutritive value of different proteins has been to provide rats with basal diets adequate in all other respects but containing no protein or amino acids. By this method, the protein element in the diet may be made the only limiting factor. As knowledge of the remaining factors in nutrition has increased, the composition of the basal non-protein rations has been subject to numerous modifications, but the principle has remained the same. By adding to such rations suitable amounts of a single protein, it has been possible to determine which are and which are not adequate for maintenance and growth.[110]

Although several investigators had previously attempted to compare the nutritive value of different proteins, the first important contributions to the subject were the classical studies of Osborne and Mendel.[111]

[109] This statement may be qualified to some extent. Mendel has stated (see also *J. Franklin Inst.*, July, 1921) that sheep may gain many pounds over considerable periods of time on a diet of starch, denitrogenized straw, inorganic salts, and urea, an exceedingly simple nitrogenous mixture that readily disintegrates to form ammonia and carbon dioxide. In the rumen, or paunch, of the sheep, as well as in that of other ruminants, there is ample opportunity for the synthesis of amino acids by bacteria. These amino acids may even be synthesized into protein, which is incorporated into the protoplasm of the bacteria. When the bacteria pass into the acid-secreting stomach and die, this protein is presumably digested and utilized in the usual manner. However, amino-acid synthesis by bacteria is not a factor in the nutrition of man and most of the higher animals. These depend on an exogenous supply of the essential amino acids. ("Nutrition: The Chemistry of Life," p. 124.)

[110] Maintenance and growth are not the only criteria employed in estimating the biological values of proteins. Methods based on the determination of the nitrogen balance have been used extensively. If a given protein contains the right assortment of essential amino acids, it will prove a more efficient nitrogen sparer than a protein of inferior biological value.

Since gain in weight is not due solely to the accumulation of protein, McCollum and Shukers proposed what they considered to be a more precise method of determining the amount of protein formed from the food. For each test nine rats of like weight and condition were used, these being divided into groups of three. One group was killed and analyzed at the beginning of the experiment. The second group was sacrificed at the end of 28 days and analyzed; the three remaining rats continued on the diet for 56 days, at the conclusion of which they too were killed and analyzed. Judgment as to the biological value of the proteins of the diet was based on the relation of the nitrogen stored to the nitrogen ingested. See E. V. McCollum and N. Simmonds, "Newer Knowledge of Nutrition," 4th edition, p. 82.

For fuller details concerning the subject the reader is referred to H. H. Mitchell and T. S. Hamilton, "The Biochemistry of the Amino Acids," Chapter X, and to the review by M. A. Boas-Fixsen, "The Biological Value of Protein in Nutrition," *Nutrition Abstracts & Revs.*, **4**, 447 (1934–35).

[111] T. B. Osborne and L. B. Mendel, "Feeding Experiments with Isolated Food Substances," Carnegie Inst. Pub., 156, Parts I and II, 1911. Osborne and Mendel, *J. Biol. Chem.*, **12**, 81 (1912); **15**, 311 (1913); **16**, 423 (1913); **17**, 325 (1914); L. B. Mendel, *Harvey Lectures*, 1914–1915, 101.

Osborne and Mendel did much to establish the value of feeding isolated food substances in nutritional studies. As milk was believed to be an adequate diet for young rats, these workers thought it desirable to include in their experimental rations a protein-free milk preparation for the purpose of supplying the necessary mineral constituents and other possibly essential ingredients. The dried "protein-free milk" constituted 28.2 per cent of the ration. The other constituents were starch 20.8, agar-agar 5.0, and fat 28.0 per cent. The proteins used in the experiments were highly purified and were supplied in liberal amount (18 per cent of the total food).

The observations of Osborne and Mendel showed that growth in rats could be secured with certain proteins but not with others. The proteins which, when fed singly in suitable concentration, proved adequate for growth included:

Proteins of Animal Origin	Proteins of Vegetable Origin
Casein (milk)	Edestin (hemp-seed)
Lactalbumin (milk)	Globulin (squash-seed)
Ovalbumin (hen's egg)	Excelsin (Brazil-nut)
Ovovitellin (hen's egg)	Glutelin (maize)
	Globulin (cottonseed)
	Glutenin (wheat)
	Glycinin (soybean)
	Cannabin (hemp-seed)

The following proteins, when fed alone, failed to induce growth:

Legumelin (soybean)	Hordein (barley)
Vignin (vetch)	Conglutin (blue or yellow lupin)
Gliadin (wheat or rye)	Gelatin (horn)
Legumin (pea)	Zein (maize)
Legumin (vetch)	Phaseolin (white kidney bean)

The failure of certain proteins to promote growth cannot be attributed to any toxic effect which they may possess; nor is the effect referable to a diminished utilization due to incomplete digestion. The evidence all points in one direction, namely, that it is fundamentally a question of amino-acid deficiency. A comparison may be made of the results obtained with foods containing either casein or gliadin as the sole protein. In their classical experiments, Osborne and Mendel demonstrated that, when casein is fed to rats, normal growth occurs, but when gliadin is the sole protein of the diet, growth occurs very slowly or not at all. Their results are represented in Fig. 37. When a comparison is made of the amino acids present in the two proteins, it is seen that casein contains all the amino acids, although the content of cystine is low. On the contrary, gliadin lacks glycine, and, as compared with casein, is poor in lysine. The absence of glycine does not constitute an actual deficiency, for this amino acid is readily synthesized by the body. The content of lysine in gliadin is apparently sufficient for maintenance and

apparently also for a slight amount of growth, but insufficient to permit normal growth. That lysine is the factor limiting the nutritional value of gliadin may be shown by supplementing such diets with this amino acid.

An even more striking illustration is to be found in the experiments (Osborne and Mendel) in which zein was fed as the sole protein. One of the curves included in Fig. 37 shows that Rat 1530 gained weight on

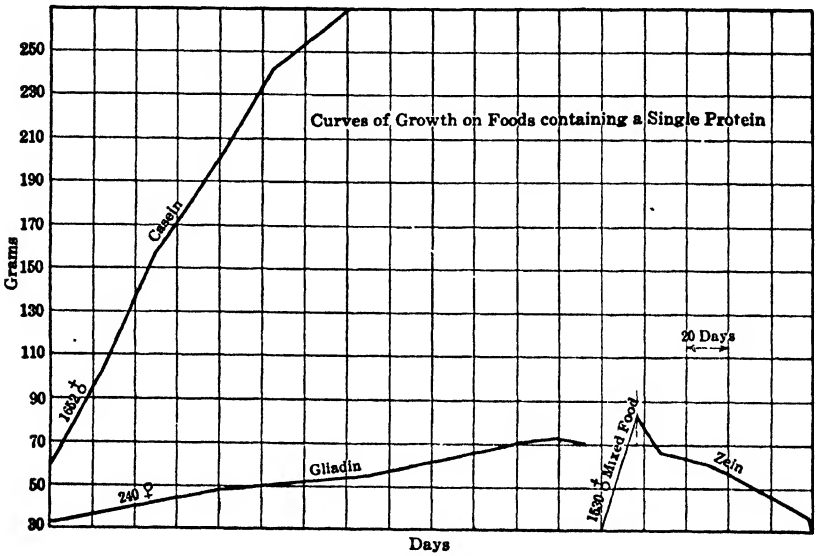


FIG. 37.—Showing typical curves of growth of rats maintained on diets containing a single protein. On the casein food (deficient in glycine) satisfactory growth is obtained; on the gliadin food (deficient in lysine) little more than maintenance of body weight is possible; on the zein food (devoid of glycine, lysine, and tryptophane) even maintenance of body weight is impossible. (After L. B. Mendel, *J. Amer. Med. Assoc.*, **64**, 1539 [1915]); "Nutrition: The Chemistry of Life," Yale Univ. Press, 1923, p. 117.)

a mixed diet, but that with the restriction of the protein element to zein there resulted at once a loss of weight. Zein is deficient in glycine, lysine, and tryptophane, the last two of which are indispensable to proper nutrition. The addition of tryptophane to the deficient diet prevented further loss of weight but did not induce any growth, whereas the addition of both tryptophane and lysine caused prompt gain in weight (Fig. 38). The dietary deficiency caused by zein may be removed in still another way, as by the addition of small amounts of lactalbumin, a protein rich in both tryptophane and lysine.[112]

[112] The inability of zein to maintain growth of young mice had been previously reported by E. G. Wilcock and F. G. Hopkins, *J. Physiol.*, **35**, 88 (1906), who found, moreover, that the addition of tryptophane (an amino acid absent from the decom-

There is another aspect to the problem. The nutritive efficiency of a protein is determined by the content of the least abundant of its component essential amino acids. This may be considered to hold also in the case of cystine, which recent work has shown to be non-essential, since it may be formed from methionine. Casein contains very little cystine (p. 118) and about 3.5 per cent methionine. Long before the existence of the latter amino acid was suspected, it was found by Osborne and Mendel that, when casein was the sole protein of the diet, normal

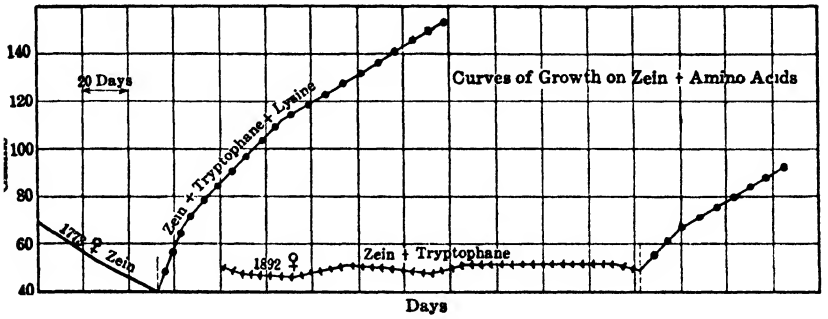


FIG. 38.—Showing the effect of the addition of the amino acids tryptophane and lysine to zein which fails to yield them. With zein alone (rat 1773) there is nutritive decline. The addition of tryptophane (rat 1892) permits maintenance without growth on foods containing zein as the sole protein. The addition of tryptophane and lysine to zein enables the animals to make considerable growth. It is interesting to note, in relation to rat 1892, that the growth of this animal was inhibited for six months without material change in its body weight. That the capacity to grow is not lost by prolonged dwarfing on imperfect food is shown by the subsequent growth of the animal when lysine was added to the food containing zein and tryptophane. (After L. B. Mendel, *J. Am. Med. Assoc.*, 64, 1539 [1915]; "Nutrition: The Chemistry of Life," p. 118.)

growth occurred in rats on an intake of 18 per cent, but when this was reduced to a lower level (9 per cent of total food intake), growth was greatly retarded. On adding cystine to the deficient ration, the nutritive efficiency was definitely improved, as shown by the marked acceleration of growth. A contrary effect resulted promptly when the cystine supplement was withdrawn from the diet. It was therefore logical to conclude that the retardation of growth which occurred on an intake of 9 per cent casein was due, not to a lack of sufficient protein *per se*, but rather to a deficiency of cystine. This explanation may still be considered as essen-

position products of zein) to such a dietary failed to influence growth favorably, though it greatly prolonged the survival period of the animals. They failed to recognize as Osborne and Mendel did later that lysine was a second limiting factor. Several years earlier, Hopkins and Cole, *J. Physiol.*, **27**, 418 (1901), had isolated tryptophane from tryptic digests. This discovery is considered by some students of the subject as having paved the way to the newer knowledge of nutrition, including the earlier research on vitamins.

tially correct, though in the light of present knowledge it may be added that the methionine contained in this ration was apparently insufficient to meet the total requirement for sulfur-containing amino acids and for sulfur metabolism. The influence of the added cystine may therefore be looked upon in part as having exerted a sparing effect on the methionine. Only when there is an abundance of methionine can a diet poor or lacking in cystine be satisfactory for growth and other bodily functions.

Work along the lines initiated in the classical experiments of Osborne and Mendel has been continued in many laboratories to the present day, and as a result much information has been obtained concerning the nutritive values of a large number of plant and animal proteins. This knowledge is of considerable importance in relation to such problems as stock and poultry raising, milk production, etc.[113]

Feeding Experiments with Protein Digests Composed of Mixtures of Amino Acids. As we have mentioned, protein digests have been used in the study of the nutritive significance of the amino acids. The first important contribution of this type was made by Abderhalden,[114] who fed to dogs amino-acid mixtures prepared from meat by digestion with appropriate enzymes. With such mixtures as the sole source of nitrogen, not only were the animals maintained in nitrogen equilibrium, but a certain number of them showed remarkable gains in weight. A dog that was fed in this way for a period of 100 days showed at the end of that time an increase in weight of 9.35 kg. Abderhalden removed both tyrosine and tryptophane from protein digests. The resulting amino-acid mixture was inadequate for maintenance or growth unless supplemented by both these amino acids.

[113] An insight into contemporary developments in this field may be obtained by consulting such recent contributions as the following: M. A. Boas Fixsen, *Nutrition Abstracts & Revs.*, **4**, 447 (1934–35); R. H. Plimmer and associates, *Biochem. J.*, **28**, 1863 (1934); H. Chick and co-workers, *ibid.*, **29**, 1702, 1712 (1935). H. Wu and associates have made an exhaustive study of the comparative value of plant and animal proteins. They made the significant observation that rats maintained on a vegetarian diet grew at a slower rate than controls ingesting a mixed diet and that this effect on the rate of growth was accentuated in successive generations. As far as could be determined the vegetarian rats showed no physical defects, nor any acute signs of deficiency; there were only an increased infant mortality and a lowered rate of growth. Wu's results suggest the possibility that a slight dietary deficiency of a degree which may not completely inhibit growth or abolish reproduction may nevertheless affect the quality of the racial stock if continued for several generations. In Wu's experiments, rats with a vegetarian ancestry of 10 to 11 generations were placed on an omnivorous diet. Growth and reproductive performance showed marked improvement in the first generation. After one generation on the omnivorous diet, the record was comparable to that of the stock omnivorous rats, thereby excluding the possibility of any hereditary difference, due to unintentional selection, between the omnivorous and vegetarian rats. See H. Wu and associates, *Chinese J. Physiol.*, **3**, 157 (1929); **5**, 71 (1931); **6**, 205 (1932); **9**, 119 (1935). See also Editorial, *J. Am. Med. Assoc.*, **105**, 438 (1935).

[114] *Z. physiol. Chem.*, **77**, 22 (1912); Abderhalden and P. Hirsch, *ibid.*, **81**, 323 (1912); *ibid.*, **83**, 444 (1913).

In a later study, Berg and Rose,[115] employing a tryptophane-free casein digest, demonstrated that when the missing amino acid was supplied at long intervals (24 or 48 hours), the effect on growth was not as beneficial as that obtained by more frequent supplementation (intervals of 6 or 12 hours). It was found that tryptophane could be replaced by synthetic 3-indolepyruvic acid, but not by 3-indolepropionic acid, or other closely related derivatives (Jackson,[116] Berg, Rose, and Marvel [117]). *d*-Tryptophane is utilized for growth in place of the naturally occurring levo form.[118]

Histidine and Arginine. As another illustration we may consider histidine and arginine. Ackroyd and Hopkins [119] hydrolyzed casein with acid and removed these amino acids from the digest. The remaining material, added to non-protein synthetic rations, was inadeqaute for the growth and maintenance of rats, but when either arginine or histidine was added, further loss of weight was avoided and growth was often resumed. The interpretation given to these observations was that histidine and arginine were interchangeable in metabolism, but that one or the other must be present in the diet.

The question of the interconversion of histidine and arginine was later subjected to critical study by Rose and Cox,[120] who compared the growth of rats upon diets in which the nitrogen was supplied (*a*) by casein, (*b*) by completely hydrolyzed casein, and (*c*) by hydrolyzed casein from which arginine and histidine had been removed. They were able to show that the rats fed upon completely hydrolyzed casein grew to maturity, though at a somewhat slower rate than animals of the same age fed upon whole casein. On the contrary, the rats that were given the arginine-histidine-free rations were neither able to grow nor to maintain body weight. Instead, there was a prompt and continuous loss of weight which could be remedied or avoided only by the addition of histidine. This part of the work, therefore, showed that *histidine is an essential component of the diet.*

Cox and Berg [121] found that *d*-histidine may serve in place of the natural *l*-modification when fed as a supplement to a histidine-deficient diet. The efficiency in promoting growth was, however, somewhat in favor of the naturally occurring enantiomorph.

As to arginine, it was found that its addition to the deficient diet exerted no perceptible influence upon growth. The animals continued to lose weight as rapidly as before the addition of this amino acid. Moreover, rations containing the minimum maintenance requirement of

[115] *J. Biol. Chem.*, **82**, 479 (1929).
[116] *Ibid.*, **84**, 1 (1929).
[117] *Ibid.*, **85**, 207, 219 (1929).
[118] C. P. Berg and M. Potgieter, *ibid.*, **94**, 661 (1931–32), V. du Vigneaud, R. R. Sealock, and C. Van Etten, *ibid.*, **98**, 565 (1932).
[119] *Biochem. J.*, **10**, 551 (1916).
[120] *J. Biol. Chem.*, **61**, 747 (1924).
[121] *Ibid.*, **107**, 497 (1934).

histidine and supplemented by large amounts of arginine were shown to be inadequate for growth. The work of Rose and Cox therefore furnished conclusive evidence that *arginine and histidine are not mutually interchangeable in metabolism.*

Concerning the nutritive importance of arginine, it seemed from the work of Bunney and Rose [122] that most, if not all, of this amino acid could be removed from a casein digest without destroying the ability of the digest to meet the growth requirements of the rat. Since there was a possibility that sufficient arginine might have remained in the digest to

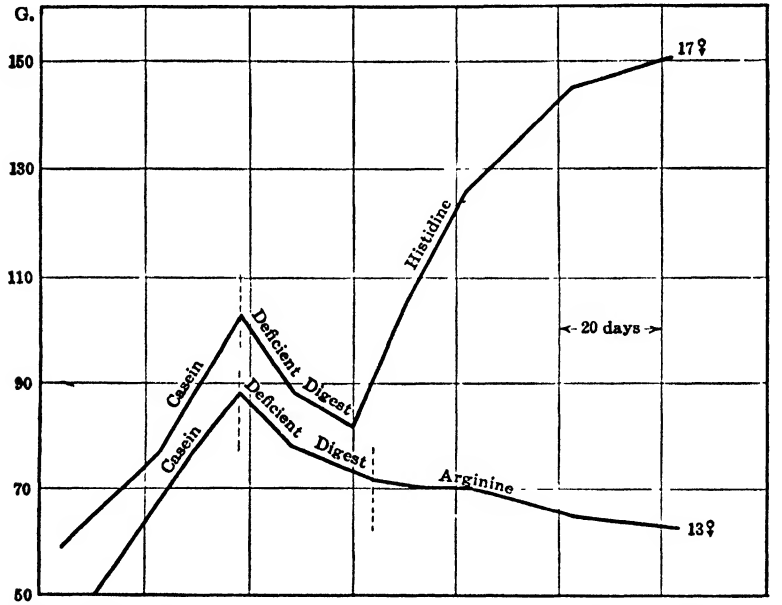


FIG. 39.—The upper curve shows the growth-stimulating effect of histidine when added to a diet deficient in histidine and arginine. The lower curve demonstrates the ineffectiveness of arginine. (After Rose.)

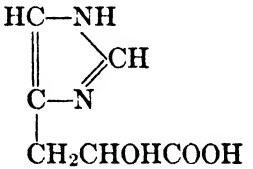
satisfy these needs, Scull and Rose [123] approached the problem from yet another angle. Rats were fed a diet of hydrolyzed casein practically devoid of arginine. At the outset of the experiment some of the control animals were sacrificed and their total arginine content determined. After 64 days on the arginine-deficient diet, during which time the animals gained considerable weight, their tissues were analyzed. Without exception the gain in tissue arginine was 2 to 3 times as large as could be accounted for by the total arginine content of the food eaten during the experimental period. This observation therefore warranted the conclusion that arginine may be synthesized in the organism of the rat

[122] *Ibid.*, **76**, 521 (1928).
[123] *Ibid.*, **89**, 109 (1930).

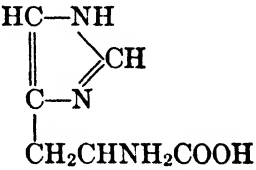
and that in this species at least it is not an indispensable component of the diet. However, it was noted later that, when arginine was excluded from the ration, growth occurred at a subnormal rate; when the arginine was restored, the rate of growth became normal. From this it seemed probable that the synthesis of arginine in the organism does not keep pace with the demands of normal growth (Rose). It has therefore been included among the essential amino acids (p. 569). According to Rose,[97] "arginine is unique in that it alone of the essential group may be excluded from the food without occasioning a *loss* in weight."

Mention may be made in this connection of the observation of Crowdle and Sherwin [124] that birds are capable of synthesizing ornithine for the detoxication of benzoic acid, the conjugation product being ornithuric acid. Because of their close chemical relation (p. 386) the synthesis of arginine from ornithine does not seem improbable.

Cox and Rose [125] were the first to demonstrate the replacement of an indispensable amino acid by a non-amino compound. These investigators showed that the addition of *dl*-β-4-imidazolelactic acid to a histidine-deficient diet caused an immediate resumption of growth (in rats) at a rate slightly slower than that induced by the equivalent quantity of histidine. Cox and Rose state, "It is evident that under the conditions of the experiments the synthetic product in question is capable of serving in place of histidine, probably through being transformed by the cells into the amino acid." Results similar to those of Cox and Rose were obtained independently by Harrow and Sherwin.[126] The close relationship between the two compounds is brought out by the following formulas:



β-4-Imidazolelactic acid



Histidine

Experiments with Known Mixtures of Highly Purified Amino Acids. We have seen that in the investigations of the type initiated by Osborne and Mendel the protein component was usually restricted to a single protein, such as casein, gliadin, zein; in these instances it was known from chemical analysis which amino acids were present and which were either lacking or present in very small amount. There was obviously no assurance of the complete absence of any one amino acid in these proteins, nor could there be any absolute certainty in the studies involving the use of protein digests that the methods employed achieved the desired end, namely the removal of all trace of even a single component. In short, there was always the possibility that a sufficient amount of the

[124] *Ibid.*, **55**, 365 (1923). [125] *Ibid.*, **68**, 781 (1926).
[126] *Ibid.*, **70**, 683 (1926).

presumably missing amino acid might have remained in the rations to satisfy the needs of the organism for growth.

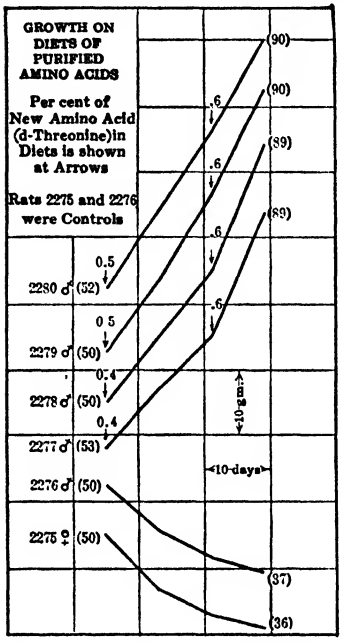


FIG. 40.— Showing normal growth in rats maintained on a ration composed of pure amino acids as the sole source of nitrogen. The numbers in parentheses denote the initial and final weights of the rats. (After McCoy, Meyer, and Rose.)

Because of these circumstances Rose [127] decided that, if further information was to be obtained concerning the nutritive significance of the amino acids, it would be necessary to "resort to the use of diets in which the proteins were replaced entirely by mixtures of highly purified amino acids." However, when rats were provided with a mixture of nineteen amino acids, including adequate amounts of all the known essential ones, they failed to grow; indeed, they lost weight. Further study, carried out with extraordinary attention to detail, disclosed that the fraction of hydrolyzed casein which was capable of fully compensating for this deficiency contained two factors: one was isoleucine; the other was eventually isolated and identified as α-amino-β-hydroxybutyric acid. Because the spatial configuration of this amino acid is analogous to that of the sugar, *d*-threose, it has been named *d*-threonine. It was found that both isoleucine and *d*-threonine were essential nutritive components.

The newly discovered amino acid was now incorporated into the amino-acid mixture,[128] and for the first time the attempt to rear animals on a ration containing pure amino acids as the sole source of nitrogen proved successful (McCoy, Meyer, and Rose [127]). This is shown by the curves in Fig. 40.

[127] W. C. Rose has summarized his work on the nutritive significance of the amino acids in *Science*, **86**, 298 (1937). More complete details are to be found in a series of papers in the *Journal of Biological Chemistry* as follows: W. C. Rose, **94**, 155 (1931–32); F. L. Catherwood and Rose, **94**, 173 (1931–32); C. T. Caldwell and Rose, **107**, 45, 57 (1934); M. Womack and Rose, **107**, 449 (1934); **112**, 275 (1935); **116**, 381 (1936); R. H. McCoy, C. E. Meyer, and Rose, **112**, 283 (1935); C. E. Meyer and Rose, **115**, 721 (1936); R. H. McCoy and Rose, **117**, 581 (1936); M. Womack, K. S. Kemmerer, and Rose, **121**, 403 (1937); W. C. Rose, *Physiol. Rev.*, **18**, 109 (1938).

[128] The composition of the amino-acid mixture used in Rose's laboratory underwent certain modifications as the work progressed. At the beginning, the various amino acids were provided essentially in the proportions in which they occur in casein (see p. 118), with the addition of isoleucine, norleucine, and cystine. In later work, increased amounts of certain of the amino acids (glycine, isoleucine, hydroxyproline, and serine) were introduced. For fuller details, the reader is referred to the publication from Rose's laboratory.[127]

These important discoveries at once opened the way to an analysis of the nutritive importance of the various amino acids with a degree of precision that was previously unattainable. In a preliminary report of the results of investigations conducted in his laboratory, Rose [97] has submitted some of the evidence for the reclassification of the essential and non-essential amino acids. The new classification is given in Table LIX.

TABLE LIX

CLASSIFICATION OF THE AMINO ACIDS WITH RESPECT TO THEIR GROWTH EFFECTS (after W. C. Rose)

Indispensable	Dispensable
Lysine	Glycine
Tryptophane	Alanine
Histidine	Serine
Phenylalanine	Norleucine
Leucine	Aspartic acid
Isoleucine	Glutamic acid
Threonine	Hydroxyglutamic acid
Methionine	Proline
Valine	Hydroxyproline
Arginine*	Citrulline
	Tyrosine
	Cystine

*Arginine can be synthesized by the animal organism, but not at a sufficiently rapid rate to meet the demands of *normal* growth.

Exclusion from the ration of any one of the amino acids listed as essential causes marked inhibition of growth and even loss of weight, whereas no apparent deleterious effects result from the exclusion of non-essential amino acids. As a crucial test of his classification Rose performed the following remarkable experiment. Rats were given a diet in which the protein was replaced by a mixture composed of only the essential amino acids; the ration was entirely devoid of all the non-essential amino acids. Under these conditions the organism was obviously forced to synthesize twelve tissue components simultaneously. It is therefore of extraordinary significance that the rats not only showed no weight loss, but on the contrary exhibited excellent growth. This demonstration is considered by Rose to be final proof that for growth only ten of the twenty-two components of protein need to be administered preformed.

It is to be appreciated that the above subdivision of the amino acids is based fundamentally on their growth importance; yet it is not improbable that this classification would be found to hold for other bodily functions. Rose has nevertheless foreseen the possibility that certain functions other than growth, notably reproduction and the detoxicating mechanism, may prove to be dependent on amino acids not included among those essential for growth.

Cystine and Methionine. Until recently it seemed that the presence of a sufficient amount of either cystine or methionine would assure the nutritional adequacy of a diet. However, from the results that have been obtained by using mixtures of pure amino acids, the conclusion has been reached by Rose and associates (Womack, Kemmerer, and Rose [127]) that cystine is not an essential amino acid, but that methionine is indispensable. The presence of an abundant supply of cystine cannot compensate for a total lack of methionine. In the absence of the latter, rats rapidly lose weight and die (Fig. 41). On the other hand, the absence of cystine is without apparent effect on maintenance and growth provided there is a sufficient supply of methionine. It should be stressed, however, that when the level at which methionine is furnished in the diet permits only maintenance or slow growth, the addition of cystine does improve greatly the quality of the diet. This explains the striking results obtained by Osborne and Mendel and by others. According to Rose, "cystine stimulates growth only when methionine is supplied in sub-optimal quantities."

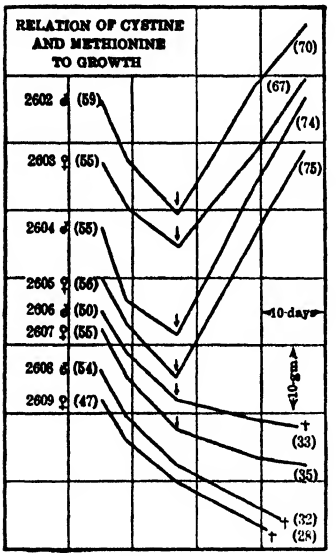


FIG. 41.—Showing that methionine is essential for growth, but that cystine is dispensable. The numbers in parentheses denote the initial and final weights of the rats. At the beginning of the experiments the animals were placed on a diet devoid of methionine and cystine. The arrows indicate the points at which dietary changes were made as follows: Rats 2602 and 2603, 0.6 per cent of *dl*-methionine plus 0.3 per cent of *l*-cystine; Rats 2604 and 2605, 1.4 per cent of *dl*-methionine; Rats 2606 and 2607, 0.6 per cent of *l*-cystine; Rats 2608 and 2609, no cystine, no methionine. Rats 2606, 2608, and 2609 died at the points indicated by the crosses. (After Womack, Kemmerer and Rose.)

Specificity of Amino Acids. We have seen that *d*-tryptophane (p. 565) and *d*-histidine (p. 565) may be utilized for growth in place of the naturally occurring enantiomorphs. According to Rose, both forms of methionine and phenylalanine promote growth, but in the case of other amino acids (lycine, valine, leucine, isoleucine and threonine), only the naturally occurring forms are physiologically active in this respect.

It will also be recalled that tryptophane and histidine may be replaced by the corresponding α-hydroxy acids. The list has now been extended by Rose to include the α-hydroxy and α-keto acids of phenylalanine, leucine, and isoleucine and of α-hydroxy-isovaleric acid for valine.

THE INDISPENSABILITY OF FAT

The independent observations of McAmis, Anderson, and Mendel [129] and of Burr and Burr [130] suggested that a certain amount of fat is essential to proper nutrition. In a carefully executed series of experiments, Burr and Burr fed rats a diet containing all the ingredients known to be necessary for the rearing of healthy animals (rats). Practically all the fat, however, had been removed from these substances, including the yeast provided as a source of the vitamin B complex, by repeated extraction with fat solvents. Vitamins A and D were furnished by the unsaponifiable matter derived from a high grade of cod-liver oil. On the fat-free diet the animals grew for a time, but soon developed symptoms indicative of a dietary deficiency disease; the skin became scaly, the tip of the tail appeared inflamed and swollen, later becoming heavily scaled, ridged, and necrotic. The hair on the back of the body became filled with dandruff and the hair about the face and neck tended to fall out. Hemorrhagic spots and sores appeared on the skin. Ovulation became irregular in the females; the males usually failed to mate; hematuria, albuminuria, and kidney lesions were prominent symptoms. Growth stopped about the time some of these symptoms became conspicuous, and death soon followed.[131]

The disease could be readily prevented or cured by the addition of linoleic or linolenic acids, either in the free form, as esters (methyl linolate), or as components of certain fats (linseed oil, corn oil, cod-liver oil, tung oil, etc.). Saturated fatty acids were without effect. Oleic acid and α-eleostearic acid, an isomer of linolenic acid, were found ineffective. According to Wesson and Burr,[130] body fat synthesized from carbohydrate provided no relief for the symptoms of the fat-deficiency disease.

Confirmatory evidence of this specific deficiency was obtained by Evans and Lepkovsky.[132] These investigators found that corn starch and rice starch were curative agents, owing to their slight but impor-

[129] *J. Biol. Chem.*, **82**, 247 (1929).

[130] *Ibid.*, **82**, 345 (1929); **86**, 587 (1930); L. G. Wesson and G. O. Burr, **91**, 525 (1931); G. O. and M. M. Burr and E. S. Miller, *ibid.*, **97**, 1 (1932).

[131] Sinclair has observed that the scaly tail condition described by Burr and Burr develops when rats are kept on coarse wire grids, or in cages with false bottoms (*Proc. Soc. Exptl. Biol. Med.*, **27**, 1059 [1929–30]); refer also to C. E. Graham and W. H. Griffith, *ibid.*, **28**, 756 (1930–31). The tendency is not so marked in rats housed on fine grids or in stock cages. In short, animals that have access to their feces, which they presumably consume under the circumstances, are much less likely to develop the characteristic scaliness. Sinclair does not believe that the difference is due to the consumption of the small amount of fatty acid contained in the feces, while Hume and Smith imply that coprophagy enables these animals to overcome some deficiency of the vitamin B complex (presumably other than vitamin B_2) (*Biochem. J.*, **25**, 300 [1931]). The condition when developed in a mild degree may be cured, according to these investigators, by the addition of whole dried yeast.

[132] *J. Biol. Chem.*, **96**, 143 (1932); **99**, 231, 237 (1932–33).

tant content of unsaturated fatty acids, presumably linoleic. Other carbohydrate foods, potato starch, hog-liver glycogen, were ineffective.

Later work revealed that successful gestation was not possible in rats kept on a fat-free diet. In about a fifth of the pregnant animals fetal resorption occurred; in others the gestation period was prolonged and there was frequent maternal mortality. The litters were small, and the offspring were undersized and born dead, or so weak that they died soon after birth. Lactation was also impaired. The outcome was essentially unaffected by the administration of increased amounts of the fat-soluble vitamins, or by supplementing the diet with *saturated* fatty acids, but astonishing improvement resulted when the essential *unsaturated* fatty acids (linoleic 80 per cent; oleic 20 per cent) were added to the ration. Males kept on a fat-free diet became sterile; this was prevented or cured by feeding small amounts of unsaturated fatty acids.

Evans and associates [133] have submitted evidence that fat exerts a sparing action on vitamin B, by which they mean that, under certain experimental conditions, the exclusion of fat from the diet raises the vitamin B requirement. They have also shown that loss of vitamin B from the tissues of rats reared without this factor is more marked when the diet is low in fat than it is when there is an abundance of fat in the rations.

That fat exerts a sparing action on vitamin B has been denied, however, by a number of workers.[134]

The realization of the probable essential nature of fat as a dietary component was a stimulus to the study of the nutritive efficiency of the constituent fatty acids (fed as esters). It was observed by Cox [135] that mixed ethyl esters of fatty acids obtained by fractionation from fat (coconut oil) promoted growth as well as the original mixture of triglycerides. With individual fatty acid esters, if fed in moderate amounts, a good state of nutrition was likewise maintained, but when the intake was increased so that it comprised as much as 77 per cent of the food calories, nutrition became definitely unsatisfactory. Ethyl stearate and palmitate were inadequately absorbed and therefore failed to support life, while ethyl and glyceryl laurate proved to be markedly toxic.

THE RÔLE OF VITAMINS IN NUTRITION

To Sir Frederick Gowland Hopkins is usually attributed the first clear statement that no animal can live upon a mixture of pure protein, fat, and carbohydrate, and that, "even when the necessary inorganic material is carefully supplied in order to supplement this diet, the animal cannot

[133] *J. Biol. Chem.*, **83**, 269 (1929); **96**, 165 (1932); **99**, 235 (1932–33); **107**, 429, 439 (1934); **108**, 439 (1935).

[134] E. Gregory and J. C. Drummond, *Vitaminforsch.*, **1**, 257 (1932); B. Sure, *Proc. Soc. Exptl. Biol. Med.*, **30**, 622 (1932–33).

[135] W. M. Cox, *J. Biol. Chem.*, **103**, 777 (1933); see also S. Lepovsky, R. A. Ouer, and H. M. Evans, *ibid.*, **108**, 431 (1935).

flourish." He recognized that, in diseases such as rickets and scurvy, dietary factors were involved that were as yet obscure, and he predicted in 1906 that the later development of the science of dietetics would deal with these complex unknown factors. This was indeed a remarkable prophecy.[136] The history of the vitamin problem is of extraordinary interest, but it is possible here only to outline a few of the steps which have led to its remarkable development. More adequate discussions are to be found in a number of works that are more specifically concerned with the subject.

The Discovery of Beriberi as a Deficiency Disease. The disease beriberi is known to have existed since ancient times in India, Japan, the Malay Peninsula, Southern China, the Philippine Islands, the Dutch East Indies, and, less prominently, in other portions of the globe. The symptoms of the disease vary somewhat, the early stages being generally characterized, however, by fatigue, mental depression, loss of appetite, and gastrointestinal disturbances. As the condition progresses there is rapid atrophy of the muscles, hypertrophy of the heart, paralysis of the lower extremities, and other evidence of multiple neuritis. In so-called "wet" beriberi, as distinguished from the "dry" form just described, there is an associated edema which is evidently related to the low plasma protein, a result of protein deficiency in the diet. In particular, the development of polyneuritis and the resulting helplessness of the victim have long attracted attention.

In 1897, Eijkman,[137] a Dutch physician and chemist working in Java, reached the conclusion that beriberi was caused by long-continued consumption of polished rice. He obtained evidence that the deficiency could be removed by the addition of the rice polishings to the diet. Moreover, he succeeded in producing experimentally a similar condition in birds by feeding polished rice, having previously observed (1896) that chickens fed largely upon the remains of the food used in the hospital developed a form of polyneuritis (polyneuritis gallinarum). Eijkman originally held the view that polyneuritis was caused by a toxin contained in the polished rice and that the curative effect of the polish-

[136] Hopkins was not alone in envisaging in a general way the existence and significance of unknown nutritional factors. The views of Pekelharing, published in 1905, were essentially the same as those reached independently by Hopkins the following year. Other students of nutrition were similarly impressed by the difficulty of maintaining animals on purified diets. Indeed, Lunin, a pupil of Bunge, is said to have recognized, as early as 1881, that substances other than protein, fat, carbohydrate, and salts were indispensable for proper nutrition.

[137] C. Eijkman, *Virchow's Arch.*, **148**, 523 (1897); **149**, 187 (1897); *Arch. Hyg.*, **58**, 150 (1906). In 1882, Takaki, a high-ranking medical officer in the Japanese navy, investigated the problem of beriberi, the incidence of which among the enlisted personnel amounted to 20–40 per cent annually. Takaki clearly recognized the relation of the disease to a one-sided diet, mainly of polished rice, and demonstrated that it could be avoided, or cured, by improvement of the diet through the inclusion in the rations of meat, fish, vegetables, and the replacement of some of the rice by barley.

ings was due to the presence of an antitoxin. Later, it was shown by Fraser and Stanton [138] that alcohol extracts of rice polishings exerted a curative effect on a beriberi patient. The problem was studied experimentally by Casimir Funk,[139] who fractionated extracts of rice polishings and obtained a crystalline product which was very active in curing and preventing polyneuritis in pigeons, and which he believed to be the physiologically active principle in preventing beriberi in man.[140] Soon after, Funk reported the isolation of the same substance from brewer's yeast. His analyses showed it to contain nitrogen in basic combination, and he therefore considered that it might be in the nature of an amine. As this principle seemed to be essential to life, he suggested the term *vitamine* for substances of this character.

The importance of Funk's discovery was overshadowed by the partly erroneous conclusion of other workers that his crystalline product was not the active principle, but an inactive substance containing a trace of the active substance. Moreover, it turned out not to be an amine. Therefore, in the original sense, the term "vitamine" was now regarded by some as a misnomer. Hopkins [141] suggested the term "accessory food factors" for the substances which Funk called "vitamines," but this designation likewise met with criticism since the word "accessory" did not imply that these components were indispensable. Ultimately it was agreed to accept the term *vitamin*.

The discovery of the importance of the water- and alcohol-soluble constituent of rice polishings as the etiological factor in beriberi stimulated many investigations of other diseases which appeared to be due to dietary deficiencies, such as scurvy, rickets, and pellagra. Since 1911, additional vitamins have been discovered. These are designated by the letters A, B, C, D, E, etc., this nomenclature being an outgrowth of a suggestion originally made by McCollum.

Vitamin A

In 1912, Hopkins [141] drew attention to certain observations that normal growth does not occur on rations consisting of purified foodstuffs, but that it does take place upon the addition to such rations of small amounts of milk. In the following year, Osborne and Mendel [142]

[138] *Lancet*, i, 733 (1910); ii, 1755 (1910).

[139] *J. Physiol.*, **43**, 395 (1911); *ibid.*, **45**, 75, 489 (1912–13); *ibid.*, **46**, 173 (1913); *Z. physiol. Chem.*, **88**, 352 (1913).

[140] A single dose of 4–8 mg. of Funk's crude crystalline product cured pigeon polyneuritis in 2–3 hours. Three compounds were isolated from yeast: I, $C_{24}H_{19}O_9N_5$; II, $C_6H_5O_2N$ (nicotinic acid); III, $C_{29}H_{23}O_9N_5$. Compound I was active, but much less than the crude product. Compounds II and III were inactive. Compounds I (3–5 mg.) + II (2 mg.) exerted the same curative action as the crude crystalline product.

[141] *J. Physiol.*, **44**, 425 (1912).

[142] *J. Biol. Chem.*, **15**, 311 (1913); **16**, 423 (1913); **17**, 401 (1914).

reported similar observations; they showed, moreover, that rats restricted to synthetic diets with lard as the source of fat frequently developed a peculiar infection of the eyes (ophthalmia or xerophthalmia), and that this condition could be remedied by the introduction of butterfat into the food. Evidently the butterfat contained something that was essential to nutrition. Somewhat later, Osborne and Mendel found that cod-liver oil likewise exerted an anti-xerophthalmic effect. In the meanwhile, McCollum and Davis [143] were independently engaged in the study of the problem, and in 1915 attributed the growth-promoting effect induced by feeding butter, egg yolk, cod-liver oil, etc., to the presence of an essential factor differing from the water-soluble vitamin obtained by Funk from yeast and rice polishings. Thus, by the work of Osborne and Mendel, McCollum, and Davis, the second vitamin was discovered. Because of its solubility in fats, it became known as fat-soluble A, in contradistinction to Funk's vitamin, which received the designation water-soluble B. In 1917, McCollum and Simmonds [144] were able to show that xerophthalmia was due specifically to a lack of the fat-soluble A vitamin. Hence the term "anti-xerophthalmic vitamin" also came to be used in designating this vitamin. At this point, it should be emphasized that as early as 1913 Osborne and Mendel fully appreciated the nature of xerophthalmia as a deficiency disease, for they referred to it as a nutritive deficiency prevalent in animals inappropriately fed, and speedily alleviated by the introduction of butterfat into the diet.

Chemical Properties of Vitamin A. Our present understanding of the chemistry of vitamin A may be partly traced back to the observation by Steenbock [145] of the apparent relation between the vitamin A potency of various plant and animal substances and the presence of the yellow pigment *carotene* (also spelled carotin).[146] Thus it was found that white corn differed from yellow corn, the former being deficient, whereas the latter was relatively rich in vitamin A. Similarly, yellow butter appeared to contain more vitamin than white butter.

At the time, several circumstances stood in the way of further rapid progress, the principal one being that the requirements for growth were imperfectly understood (vitamin D had not yet been discovered). Inasmuch as the method of vitamin A assay employed growth as a criterion, the results obtained in different laboratories showed many inconsistencies and were frequently at variance.

These handicaps, however, were overcome in time. With the intro-

[143] *Ibid.*, **15**, 167 (1913); **19**, 245 (1914); **20**, 614 (1915); **23**, 231 (1915).

[144] *Ibid.*, **32**, 181 (1917).

[145] *Science*, **50**, 353 (1919); H. Steenbock and P. W. Boutwell, *J. Biol. Chem.*, **41**, 81 (1920); Steenbock and E. G. Gross, *ibid.*, **41**, 149 (1920); Steenbock and M. T. Sell, *ibid.*, **51**, 63 (1922).

[146] The principal pigment of yellow corn is not carotene, but a closely related pigment, cryptoxanthine (p. 578).

duction of improved biological methods of assay and with the development of certain chemical and physical tests the way was opened to the further pursuit of the problem. With arsenic trichloride vitamin A gives a characteristic ultramarine blue color which changes to purple and then gradually fades (Rosenheim and Drummond).[147] A more lasting color is obtained with antimony trichloride ($SbCl_3$), as shown by Carr and Price.[148] Originally it was thought that these reactions were specific for vitamin A, but it has been found recently that various plant pigments react with these reagents.[149] Nevertheless, the antimony trichloride test proved useful, not only for the detection of vitamin A in various substances, but also in the study of the relation between it and carotene.

With vitamin A, antimony trichloride yields an intense blue color, exhibiting an absorption band 610–630 mμ (millicrons). Carotene, on the other hand, reacts to give a greenish blue color, exhibiting a characteristic absorption band at 590 mμ. Another striking property is that discovered by Takahashi.[150] He found that oils rich in vitamin A give an absorption band in the ultraviolet, at 328 mμ (3280 Å). This band is not exhibited by carotene. Still another difference is that carotene is intensely yellow in color, while relatively pure preparations of vitamin A frequently have little or no color. In short, on the basis of these properties there was at first no reason to suppose that any relation existed between carotene and vitamin A.

However, in 1928, Euler [151] prepared pure carotene from carrots and found it to be very effective in supplementing vitamin A deficient diets. The daily administration of only 0.005 mg. was sufficient to restore promptly the growth of rats suffering from vitamin A deficiency. The efficacy of carotene as a substitute for vitamin A both in promoting growth and in curing ophthalmia in rats was soon confirmed by others, notably by Moore.[152] This investigator found, moreover, that the liver oil of a rat depleted of vitamin A did not give a reaction with antimony trichloride. When highly purified carotene, or carrots, were fed, not only did the symptoms of vitamin A deficiency clear up, but most remarkable was the fact that the liver oil now exhibited an absorption band at 328 mμ and reacted with antimony trichloride to give the characteristic color reaction and absorption spectrum of vitamin A. No other conclusion seemed possible than that carotene, or some part of it, had been transformed in the body into vitamin A.

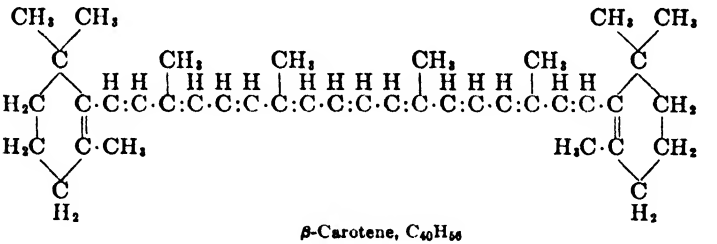
Evidence rapidly began to accumulate in other laboratories substantiating these results. It was found that a large variety of food sub-

[147] *Biochem. J.*, **19**, 753 (1925).
[148] *Ibid.*, **20**, 497 (1926).
[149] H. von Euler, P. Karrer, and Rydbom, *Ber.*, **62**, 2445 (1929).
[150] *Tokyo Inst. Phys. Chem. Research*, **3**, 81 (1925).
[151] *Biochem. Z.*, **203**, 370 (1928).
[152] *Biochem. J.*, **23**, 803 (1929); **24**, 692 (1930); **25**, 275 (1931).

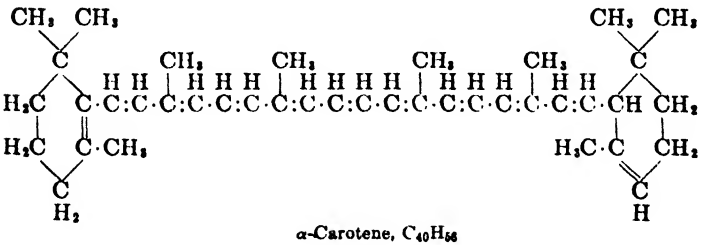
stances containing carotene, when consumed, were sources of vitamin A. The transformation is believed to occur in the liver, which is also the main site of vitamin A storage, although accumulation of the vitamin may occur in other organs (lung, kidney).

Not all the carotene administered is necessarily absorbed, or converted into vitamin A; indeed the transformation occurs rather slowly. Thus, when carotene is fed to a cow, it is partly converted into vitamin A, a portion of unchanged carotene being found in the liver (Moore). Likewise, in milk and butter, carotene and vitamin A coexist in varying proportions (Baumann and Steenbock).[153] Colostrum is rich in respect to both carotene and vitamin A.[154]

Carotene exists in at least three isomeric forms, as disclosed by Karrer and associates [155] and independently by Kuhn and Lederer.[156] β-Carotene is optically active; α-carotene is optically inactive. A third pigment, γ-carotene, was isolated by Kuhn and Brockman.[157] The pigment of yellow corn is cryptoxanthine; it is closely related to carotene and was identified by Kuhn and Grundman.[158] All these are precursors of vitamin A. One molecule of β-carotene yields two molecules of the vitamin; the other pigments are potentially capable of forming vitamin A, molecule for molecule. These pigments, or provitamins, are represented by the following structural formulas:

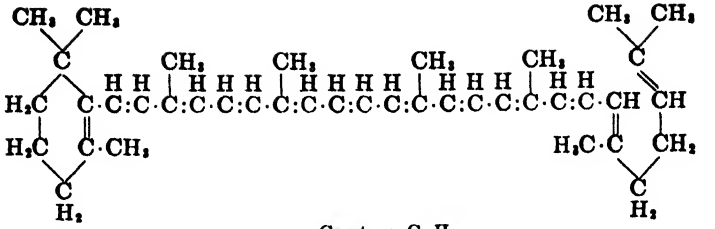


β-Carotene, $C_{40}H_{56}$

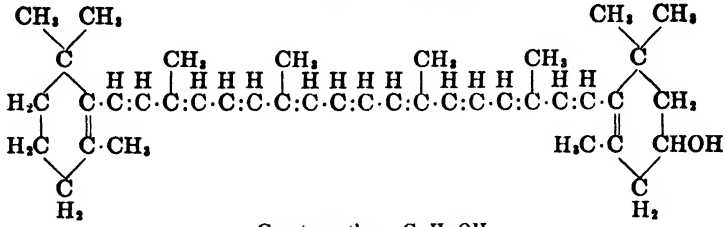


α-Carotene, $C_{40}H_{56}$

[153] *J. Biol. Chem.*, **101**, 547 (1933) **105**, 167 (1934).
[154] W. J. Dann, *J. Biochem.*, **27**, 1998 (1934); J. Semb, C. A. Baumann, and H. Steenbock, *J. Biol. Chem.*, **107**, 697 (1934).
[155] *Helv. Chim. Acta*, **14**, 614 (1931).
[156] *Ber.*, **64**, 1359 (1931).
[157] *Ber.*, **67**, 885 (1934).
[158] *Ber.* **67**, 593 (1934).



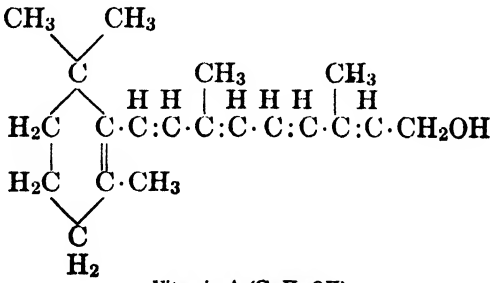
γ-Carotene, $C_{40}H_{56}$



Cryptoxanthine, $C_{40}H_{55}OH$

These developments stimulated intensive activity in this field of investigation, embracing not only the relation of carotene to vitamin A, the isolation of the pure vitamin, but also the chemistry of the carotinoid pigments, their derivatives, and related compounds.[159]

Brief reference may now be given to the isolation of vitamin A. This was accomplished independently in several laboratories. Karrer, Morf, and Schöpp [160] obtained a very potent preparation from halibut-liver oil. Moore [161] obtained vitamin A from the liver oil of rats and pigs that had been fed rich sources of carotene, such as red palm oil. Heilbron, Drummond, and co-workers [162] obtained their concentrate from cod-liver oil. The following formula for vitamin A, proposed by Karrer, is now generally accepted.



Vitamin A ($C_{20}H_{29}OH$)

[159] The student is referred to the reviews by P. Karrer and A. Helfenstein, *Ann. Rev. Biochem.*, **1**, 551 (1932); **2**, 397 (1933); consult also R. Kuhn, *ibid.*, **4**, 479 (1934), and F. S. Spring, *Ann. Repts., Chem. Soc., London*, **32**, 291 (1935).

[160] *Helv. Chim. Acta*, **14**, 1036, 1431 (1931).

[161] *Biochem. J.*, **25**, 2131 (1931).

[162] *Ibid.*, **26**, 1178 (1932).

The vitamin is a viscous, somewhat yellowish oil. It distils in vacuo with partial decomposition. It is an alcohol and forms esters. As is evident from the formula it is an unsaturated hydrocarbon, consisting of a β-ionone ring and a side chain containing 4 double bonds. Crude vitamin A resists catalytic hydrogenation; the pure product is more readily reduced. This difference in behavior is also true for oxidation. As it exists in natural foods and other products, vitamin A resists destruction much more than the isolated vitamin. In certain solvents (ethyl alcohol, ethyl acetate, alcoholic KOH), vitamin A is relatively stable to oxidation in air. Its stability in natural products has been attributed to the presence of antioxidants.

Nutritional Significance of Vitamin A.[163] The *growth-promoting* properties of vitamin A were the first to be recognized and studied. Soon, however, it was disclosed that deficiency with respect to this vitamin may result in a well-defined infection of the eyes, which begins in the lids and later involves the cornea. Apparently because of the impairment of the tear glands the cornea becomes dry and subsequently opaque owing to the formation of a horny layer, a process called *keratinization* or *cornification.* The disease is termed *xerophthalmia.*[164]

Medical literature contains many references to the prevalence of eye disease in communities in which malnutrition may be supposed to exist. Among Japanese children, Mori [165] found the incidence of xerophthalmia to be very high, especially in times of famine. It is interesting to note in this connection how even empirical methods often prove to be of lasting value, for Mori's treatment consisted in feeding his patients cod-liver oil and chicken livers. At one time a similar situation prevailed in Denmark. Owing to the demands of the butter industry, the use of skim milk in infant and child feeding was so general among the poor that xerophthalmia became very prevalent. Bloch [166] studied the condition exhaustively and ascribed it to fat deficiency. Butter and cod-liver oil proved effective in treating the disease. In the light of our present knowledge, the conclusion is justified that, both in Mori's and Bloch's clinical studies, the conditions were largely due to a deficiency of vitamin A. To remedy the situation in Denmark, the government

[163] The clinical features of vitamin A deficiency have been reviewed by G. B. Eusterman and D. L. Wilbur, *J. Am. Med. Assoc.*, **98**, 2054 (1932). The reader should also consult S. B. Wolbach, "The Pathologic Changes Resulting from Vitamin Deficiency," *J. Am. Med. Assoc.*, **108**, 7 (1937); see also O. Klotz and W. L. Holman, "Recent Work on the Tissue Changes in Vitamin A Deficiency," *Am. J. Med. Sci.*, **192**, 409 (1936).

[164] Yudkin (*J. Am. Med. Assoc.*, **79**, 2206 [1922]) and Yudkin and Lambert (*Proc. Soc. Exptl. Biol. and Med.*, **19**, 375 [1922]) have made a study of the pathology of xerophthalmia. Of considerable importance, too, are Pillat's observations, *Nat. Med. J. China*, **15**, 614 (1929); *Arch. Ophth.*, **2**, 256, 399 (1929).

[165] *Jahrb. Kinderheilk.*, **59**, 175 (1904); *J. Am. Med. Assoc.*, **79**, 197 (1922).

[166] *J. Hyg. (Cambridge)*, **19**, 283 (1921).

found it necessary to limit the exportation of butter, thereby increasing its consumption within the country. This measure is said to have decreased, to a very marked extent, the incidence of xerophthalmia among the poorer classes.

In the United States xerophthalmia is rare. It is frequent in China, India, and Java. It is said that in Yucatan, where poverty is widespread, one child in every five of the poor classes suffers from xerophthalmia.

Keratinization of the cornea, which is a comparatively late manifestation, exemplifies the rather generalized effect of vitamin A deficiency on epithelial structures. Clinicial and experimental work has brought to light certain specific pathological changes which occur in the skin, stomach and intestines, urogenital system, and elsewhere and has led to the conclusion that one of the important functions of vitamin A is to maintain the integrity of epithelium. As a result of vitamin A deprivation, epithelial cells tend to atrophy and, irrespective of their original form or function, they are eventually replaced by stratified keratinized epithelium. Wolbach [163] has dwelt at length on the distribution of the keratinizing metaplasia. In human infants suffering from vitamin A deficiency, it has been found in the conjunctiva, mucosa of the nares, accessory sinuses, trachea, bronchi, pancreas, renal pelvis, ureters, and elsewhere. Wolbach states, "the early effect of deficiency on the respiratory mucosa is a satisfactory explanation of the frequency, severity and persistence of the pneumonias that have been in most instances responsible for death in vitamin A deficient infants."

This brings up the question of vitamin A deficiency as a predisposing factor in disease, particularly infections. Critical students of nutrition do not pretend that a relation has been established between vitamin A and the immunological mechanism of disease, or that vitamin A is a specific agent in preventing certain infections. The relation is a broader one, having to do with the effect of vitamin A on resistance in general. As has been stated by Sherman,[167] "on the more comprehensive (if technically less precise) question, there is much positive evidence that the level of intake of vitamin A does (whether directly or indirectly, primarily or secondarily) influence the frequency, or severity, or duration, of infectious disease—not specifically of any one infection alone, and also not equally of all infections."

Evidence has been submitted by Zimmerman,[168] de Aberle,[169] Mellanby,[170] and others that prolonged vitamin A deficiency produces definite lesions in the central nervous system.

The relation of vitamin A to the metabolism of the visual purple of

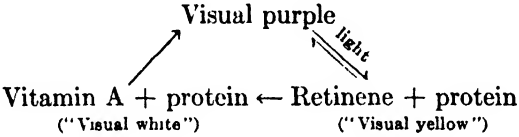
[167] Chemistry of Foods and Nutrition, 1937 edition, p. 371. See also E. C. Robertson, "The Vitamins and Resistance to Infection," *Medicine*, **13**, 123 (1934).

[168] *J. Exptl. Med.*, **57**, 215 (1933).

[169] *J. Nutrition*, **7**, 445 (1934).

[170] *J. Path. Bact.*, **38**, 391 (1934).

the retina has been recognized for a number of years.[171] It has been found that the retina and the other pigment layers of the eye contain comparatively large amounts of the vitamin. From the important work of Wald,[172] particularly on the pigments of the frog's eye, it appears that vitamin A is the precursor of visual purple. The transformation can occur only in the intact eye, but the other reactions to be described occur both in the intact animal and in the isolated retina. It seems that light causes the conversion of the visual purple into so-called "visual yellow" which is composed of the pigment *retinene* and a protein. The retinene liberated in the process can be utilized either in the restitution of the visual purple or in the formation of vitamin A and other colorless products. The reactions involved in the visual cycle have been represented as follows:



According to Wald, the visual purple system expends vitamin A and is accordingly dependent on the diet for its replacement.

In view of the foregoing it is of extraordinary interest that night blindness or "hemerolopia" has for a long time been associated with vitamin A deficiency. In this condition there is difficulty or inability to see in dim light. Night blindness in its more obvious form has been very common among the inhabitants of Newfoundland, Labrador, Russia, Japan, certain parts of India, and other places. It was evidently known to the ancients, for Hippocrates is said to have recommended, among other things, liver as a therapeutic measure. In recent years Jeans [173] has called attention to the fact that a moderate degree of night blindness, or at any rate reduced ability to dark adaptation, is very common among children. He has related this to an inadequate intake of vitamin A and has reported improvement when the intake of this vitamin was increased, even though the diet was not changed materially otherwise.

Another manifestation of vitamin A deficiency is the tendency to form calcium phosphate concretions in the urinary tract. This was first described by Osborne and Mendel [174] and has since been confirmed by van Leersum and others.[175] This condition of urolithiasis occurs exten-

[171] L. S. Friedericia and E. Holm, *Am. J. Physiol.*, **73**, 63 (1925); A. M. Yudkin, M. Kriss, and A. H. Smith, *ibid.*, **97**, 611 (1931); G. Wald, *J. Gen. Physiol.*, **18**, 905 (1935); K. Tansley, *Biochem. J.*, **30**, 839 (1936).

[172] *J. Gen. Physiol.*, **19**, 351, 781 (1935–36).

[173] P. C. Jeans and Z. Zentmire, *J. Am. Med. Assoc.*, **102**, 892 (1934); *ibid.*, **106**, 996 (1936); P. C. Jeans, E. Blanchard, and Z. Zentmire, *ibid.*, **108**, 451 (1937).

[174] *Ibid.*, **69**, 32 (1917).

[175] *J. Biol. Chem.*, **76**, 137 (1928).

sively in the tropics and the Far East, which suggests the possibility of its being associated with a dietary low in fat and fat-soluble vitamins (Mendel).

The new-born mammal, including the human infant, has a low vitamin A reserve. It cannot be materially improved by feeding an abundance of vitamin A or its precursor, carotene, to the mother during the period of gestation. Although it is possible to impoverish the milk by depriving the lactating animal of vitamin A, it is impossible to enrich it greatly by providing an overabundance of the vitamin. During the suckling period, the young animal does, however, accumulate a small reserve. It has been suggested by Dann [176] that in human beings direct feeding of vitamin A to the infant must be employed to build up its reserves. In young animals in which a considerable store has been laid down by feeding cod-liver oil, subsequent deprivation does not result at once in the cessation of growth or in the development of other signs of vitamin A deficiency. Indeed growth may continue for a considerable period.[177]

Standardization of Vitamin A. The international vitamin A unit is the vitamin A value equivalent to 0.6 γ (0.0006 mg.) of pure β-carotene. It is now possible to determine vitamin A potency spectrophotometrically by measuring the intensity of absorption at 328 mμ (3280 Å). The material to be examined is extracted in ethyl alcohol or in cyclohexane. In the standardization of cod-liver oil, extracts of the unsaponifiable residue are used; the determination in halibut-liver oil is frequently made without preliminary saponification.

The blue color reaction of vitamin A with antimony chloride has found considerable application in the semi-quantitative evaluation of the vitamin A potency of oils and other materials.

Of the biological methods, the most important one depends on determining the increase in weight of young rats.[178] Rats 21 to 29 days old, deprived of vitamin A in the rations, lose weight. The loss is minimized by the addition of small amounts of vitamin A, or its precursors; larger amounts enable growth. The so-called Sherman unit of vitamin A value is the amount which when fed daily just suffices to support a rate of gain of 3 grams per week in a standard test animal during an experimental period of 4 to 8 weeks.

One Sherman unit is equivalent to about 1.4 international units.

Distribution of Vitamin A in Foods. Milk, cream, butter, cheese, egg yolk, and the green leaves of plants are among the most abundant

[176] *Biochem. J.*, **26**, 1072 (1932).

[177] For data on the vitamin A reserve in health and disease, consult T. Moore, *Biochem. J.*, **31**, 155 (1937); J. B. Ellison and T. Moore, *ibid.*, **31**, 165 (1937); see also A. W. Davies and T. Moore, *ibid.*, **29**, 147 (1935).

[178] For more complete details the reader is referred to K. H. Coward, *Nutrition Abstracts & Revs.*, **4**, 705 (1934); consult also B. L. Oser in Hawk and Bergeim's "Practical Physiological Chemistry," 11th edition, 1937, Chapter XXXV.

sources of vitamin A. Cod-liver oil is an excellent source, but halibut-liver oil has been found to contain even more vitamin (this is also true of vitamin D).[179] For the vitamin A values of foods the reader should consult Sherman, p. 167.[179a] The following are selected data (expressed in international units per 100 grams) of some common articles of diet. In some instances the range of observed values is indicated; in others, the mean and the probable error of the mean are given.

	International Units		International Units
Apples	105±8	Egg Yolk	5000±294
Apricots	4480–12,040	Kale	36,260±840
Beans, dry kidney	0	Liver	10,200–11,200
" string, fresh	580–1400	Milk	292±12
Beef	21–112	Oatmeal	0
Bread	15	Oils, vegetable, refined	0
Broccoli, flowers	9660–12,600	Potatoes	56
" leaves	25,000–42,500	Spinach	20,700±1500
Butter	5060±180	Sweet potatoes	3600±520
Cabbage, English, green	880	Tomato, skins and seeds removed	1100±140
Carrots	4350±460	Wheat	0
Cod-liver oil	100,000–250,000	Yeast, dry, bakers'	0

Vitamin A Requirement. More precise information is needed concerning the vitamin A requirement of man in health and disease. The estimates for the normal individual are somewhat at variance, but it is probably safe to place the adult requirement at about 5000 international units. Infants and children probably need about 3000 to 4000 units, while the pregnant or lactating woman should receive at least 9000 units. It is therefore to be realized that a one-sided diet, composed of bread, beans, oatmeal, and vegetable oils, would inevitably result in vitamin A deficiency. However, even such a diet may be corrected, at least from the standpoint of vitamin A, by the addition of such articles as liver, kale, spinach, or carrots, and certainly by giving sufficient cod-liver oil.

The Antineuritic Vitamin, or Vitamin B_1, and Other Components of the "Vitamin B Complex"

The early history of the discovery of vitamin B also traces the beginnings of our present knowledge of the deficiency diseases and was therefore used in introducing the subject. We have noted that rice polishings contain a water-soluble principle, designated as vitamin B, which is capable of preventing and curing polyneuritis in pigeons and beriberi in

[179] The vitamin A potency of halibut-liver oil has been found to be 41,000 I. u. per gram, compared to 1.000–2.500 I. u. per gram of cod-liver oil. Bills and associates have determined the vitamin A content of a variety of fish-liver oils. *J. Nutrition*, **13**, 435 (1936). The following are illustrative data (I. u. per gram of oil): bluefin tuna, 84,000; California mackerel, 88,000; swordfish, 250,000; black sea-bass, 520,000; cabrilla, 170,000; sardine, 2,700.

[179a] Consult also E. P. Daniel and H. E. Munsell, "Vitamin Content of Foods," U. S. Dept. Agr. Misc. Pub. No. 275.

man. Much of the work that followed its discovery was concerned with its distribution in plant and animal tissues and with the study of the various manifestations resulting from its lack in the diets of man and beasts. It was soon recognized that vitamin B apparently exerted profound effects on growth, gastrointestinal motility, appetite, lactation, the integrity of nerve tissue, carbohydrate metabolism, etc.

In time it was also discovered that, in many foods containing vitamin B, there was an additional factor. Emmett and Luros [180] found that the antineuritic factor in unmilled rice was destroyed by autoclaving at 120° C. and 15 pounds pressure for 2 to 6 hours. There remained, however, a growth-promoting, water-soluble principle. Smith and Hendrick [181] discovered that autoclaved yeast exhibited no antineuritic effect, but was growth-promoting. Mitchell [182] was perhaps the first to call attention to the discrepancy in the distribution in foods of the antineuritic principle and the other water-soluble constituent which apparently was only growth-promoting. These and many other observations led to the conclusion that besides the antineuritic vitamin there was another water-soluble vitamin. This was named vitamin B_2 by British biochemists; in America it was usually designated by the letter G.

Later work made it seem probable that there existed still other components of the vitamin B complex, B_3, B_4, B_5, B_6, etc., but up to the present their chemical identity and nutritional significance have not been completely elucidated. We shall return to a brief consideration of these factors, as well as of vitamin B_2 (G), shortly, and will now direct our attention to the antineuritic vitamin.

In as brief a discussion as this it is obviously impossible to follow the enormous amount of work that has been done on the subject of vitamin B_1. We shall therefore confine ourselves to a few brief statements of its chemical constitution and properties, nutritional significance, method of assay, distribution in foods, and the approximate requirement for man.

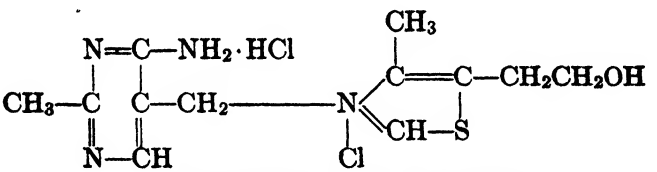
Nomenclature. As no uniform system of terminology has been adopted for the compounds of the vitamin B complex, the question of nomenclature is a perplexing one. In the United States, the antiberiberi (antineuritic) vitamin has been commonly designated by the letter B, whereas in the British terminology "B" refers to the complex and "B_1" to the antiberiberi vitamin. Many American writers have used "B" and "B_1" interchangeably. In drawing on the vitamin literature of American, British, German and other workers, it becomes comparatively difficult, therefore, to be entirely consistent from the standpoint of nomenclature.

The American Society of Biological Chemists and the American Institute of Nutrition have approved but have not yet adopted the recommendations of the Committee on Vitamin Nomenclature to aban-

[180] *J. Biol. Chem.*, **43**, 265 (1920).
[181] *U. S. P. H. Reports*, **41**, 201 (1926).
[182] *J. Biol. Chem.*, **40**, 399 (1919).

don the term "B" and to designate the antineuritic vitamin as B_1. It has also been recommended and approved, but not adopted, that the designations "B_2" or "vitamin G" be no longer used and that the term "riboflavin" be employed for the compound identified as "6,7 dimethyl-9-(*dl'*-ribityl)-isoalloxazine."

Chemical Constitution and Properties of Vitamin B_1. Jansen and Donath [183] made an important contribution to the subject when they isolated the vitamin in crystalline form (as the hydrochloride), even though they were at first ignorant of its composition. This was determined several years later by Windaus and co-workers [184] to be $C_{12}H_{17}N_4OS$. It is of interest that a number of other investigators engaged at the time in vitamin B research attained the same goal of isolating the vitamin and determining its formula, at least approximately, almost simultaneously. The final steps in establishing the chemical constitution of vitamin B_1 were achieved by Windaus, Tscherche, and Grewe,[185] and especially by Williams,[186] who with his associates provided proof by synthesizing it (as the bromide hydrobromide). The molecular configuration of the antineuritic vitamin, according to Williams, is represented by the formula:



Vitamin B_1 chloride hydrochloride or Thiamin
$C_{12}H_{17}N_4OSCl \cdot HCl$ (The antineuritic vitamin)

From this it is seen that vitamin B_1 is 2-methyl-5-(4-methyl-5-β-hydroxyethyl-thiazolium chloride)-methyl-6-aminopyrimidine hydrochloride. The naturally occurring vitamin and the synthetic product have identical properties both chemically and physiologically. With doses of 6γ, cures of polyneuritis were uniformly effected in rats kept on a vitamin-B_1-free diet. The crystalline vitamin is soluble in water, acetic acid, and 70–80 per cent alcohol. In acid or neutral solution very little destruction occurs at 100°, but above this temperature the vitamin gradually decomposes. The chloride hydrochloride melts at 248–250° C. The vitamin exhibits absorption bands at 232 and 267 mμ.

Nutritional Significance of Vitamin B_1.[187] Beriberi is a condition of severe vitamin B_1 deficiency. It has been reproduced experimentally

[183] *Verslag. Akad. Wetensch.*, Amsterdam, **35**, 923 (1926).
[184] *Z. physiol. Chem.*, **204**, 123 (1932).
[185] *Ibid.*, **237**, 98 (1935).
[186] *J. Am. Chem. Soc.*, **57**, 229 (1935); J. K. Cline, R. R. Williams, and J. Finkelstein, *ibid.*, **59**, 1052 (1937); Williams, *Ind. Eng. Chem.*, **29**, 980 (1937).
[187] This subject has been reviewed by H. D. Kruse and E. V. McCollum, *J. Am. Med. Assoc.*, **98**, 2201 (1932) and by Geo. R. Cowgill, *ibid.*, **98**, 2282 (1932).

in birds, notably the pigeon, and in the rat. The conspicuous manifestation is multiple peripheral neuritis, hence the designation *polyneuritis*. Both the clinical and experimental forms of this condition are evidently the result of shortage rather than complete absence of the antineuritic vitamin.[188] Sherman and Sandels [189] in experiments on rats found that when the allowance of vitamin was too small to maintain weight, but sufficient to prolong life, the classical symptoms of polyneuritis appeared regularly. Increasing the allowance sufficiently to maintain weight was attended by a subacute type of polyneuritis. When the animals were completely deprived of vitamin B_1 death resulted early and before apparent symptoms of polyneuritis developed.

Degeneration of the myelin sheathes of the peripheral nerves (sciatic median, ulnar) and of the brachial plexus has been found in dogs suffering from avitaminosis B.[190] Lesions have also been noted in the ganglion cells of the brain, cerebellum, spinal cord, and dorsal root ganglia, but it remains to be proved that these pathological changes are due specifically to vitamin B deficiency and not to the consequences of severe undernutrition or starvation. However, it is generally conceded that vitamin B deficiency exerts a profound effect on the physiology of the neurons.

Effect on Appetite. An outstanding symptom of vitamin B deficiency is the loss of appetite, or *anorexia*. Osborne and Mendel had observed in their earlier nutritional studies that animals deprived of what are now recognized as the water-soluble vitamins lost the desire to eat. This sympton was examined more closely by Karr [191] in Mendel's laboratory, using the dog as the experimental animal. He found that "some relation exists between the desire to partake of food and the amount of the so-called water-soluble B vitamin ingested." These findings were confirmed and greatly extended by Cowgill and his pupils, whose detailed studies over a period of years have definitely established the specificity of the effect of vitamin B (B_1) on appetite, not only in experimental animals (mouse, rat, pigeon, dog), but in man as well. Indeed this vitamin seems to play a dominant rôle in the maintenance of appetite, a fact which is likely to receive increasing recognition and application in clinical medicine.

Effect on Gastric Motility. Inasmuch as the physiological basis of the hunger sensation is recognized to be the contractions of the empty stomach, attention has been directed to the gastrointestinal tract in the endeavor to explain the effect of vitamin B_1 on appetite. Cowgill and co-workers [192] observed no pronounced change in gastric motility in the

[188] W. H. Sebrell and E. Elvove, U. S. P. H. Reports, **46**, 917 (1931); see also K. O. Elsom, *J. Clin. Investigation*, **14**, 40 (1935).

[189] M. R. Sandels, *J. Nutrition*, **2**, 409 (1930); H. C. Sherman and Sandels, *ibid.*, **3**, 395 (1930–31).

[190] H. M. Zimmerman and E. Burack, *Arch. Pathol.*, **13**, 207 (1932); see also S. B. Wolbach, *J. Am. Med. Assoc.*, **108**, 7 (1937).

[191] *J. Biol. Chem.*, **44**, 255 (1920).

[192] *Am. J. Physiol.*, **77**, 389 (1926); **91**, 531 (1932).

early stages of vitamin B deficiency, but the later stages were characterized by marked atony, not only of the stomach, but of the intestinal tract as well. The injection of vitamin B concentrate was followed by a sharp improvement in gastrointestinal motility.

Among the reported changes in severe vitamin B deficiency is the development of lesions in the gastro-intestinal tract,[193] and depressed gastric and pancreatic secretion, accompanied by reduced enzymatic activity (lipase, etc.).[194]

The lowered food consumption resulting from the loss of appetite in vitamin B deficient animals is obviously an important factor hindering growth. This has rendered difficult the differentiation of the specific effect of the vitamin on growth. The problem has been considerably clarified by the experiments of Sure,[195] in which he found that rats deprived of the antineuritic vitamin lost weight and died, while their litter-mates consuming similar amounts of food (considerably less than the normal intake), but provided with a liberal amount of vitamin B, survived for a long time, and even grew. Accordingly, it has been concluded that vitamin B produces growth in two ways: (*a*) it stimulates the appetite so that there is an increase in food consumption; (*b*) it produces a specific influence on growth, unrelated to the plane of nutrition, and is therefore essential for growth.

Lactation. The relation of vitamin B to lactation is of considerable interest. Macy [196] found that the lactation requirement of vitamin B is about 3–5 times greater than for growth. This extraordinary need and the apparent dissipation of the vitamin have been confirmed by Sure,[197] who on further investigation of the problem reached the conclusion that vitamin B exerts a specific effect on lactation. To secure successful lactation and prevent infant mortality in rats an adequate supply of vitamin B must be assured.

As stated elsewhere, Macy's studies have shown that milk is deficient in its vitamin B content. Although this is subject to variation and tends to be even lower than usual when the maternal diet is restricted, it cannot be increased materially by supplementing the diet even with such rich sources as yeast. Many students of the subject therefore share the opinion that at best the supply of vitamin B furnished to the nursing infant is barely adequate. In the case of mothers subsisting on a deficient diet, the situation for the infant is certainly critical.

[193] R. McCarrison, *Brit. Med. J.*, **2**, 36 (1919); **1**, 822 (1920). B. Sure and H. S. Thatcher, *Arch. Path.*, **16**, 809 (1933).

[194] C. J. Farmer and H. E. Redenbaugh, *Am. J. Physiol.*, **75**, 45 (1925–26); see also B. Sure, M. C. Kik, and K. S. Buchanan, *J. Biol. Chem.*, **108**, 19, 27 (1935).

[195] *J. Biol. Chem.*, **97**, 133 (1932); compare with W. H. Griffith and C. E. Graham, *ibid.*, **97** (Proc.) vii (1932).

[196] *Ibid.*, **73**, 189 (1927).

[197] *Ibid.*, **78**, 685 (1928); B. Sure and D. J. Walker, *ibid.*, **91**, 69 (1931); Sure and M. E. Smith, *J. Nutrition*, **5**, 147 (1932).

Where poverty and poor nutrition are widespread, in the Far East and elsewhere, infantile beriberi is prevalent.

Heart. Hypertrophy of the heart is a conspicuous finding in vitamin B_1 deficiency. The cause is not fully understood, but it seems to be associated with marked retention of water in the musculature. The administration of vitamin B_1 causes a rapid return of the heart to normal size. Drury and co-workers [198] have made the remarkable observation that deprivation of vitamin B in rats leads to severe bradycardia (slowing of the pulse). This can be readily cured by the injection of vitamin B concentrate. It has been suggested that the lowered heart rate may be due to the accumulation of lactic acid in the heart.[198a]

Relation to Carbohydrate Metabolism. Increasing attention has been directed recently to the question of the significance of vitamin B_1 in carbohydrate metabolism and tissue respiration, especially in the brain. The accumulation of lactic acid and pyruvic acid has been noted in brain, heart, blood, and cerebrospinal fluid in vitamin B deficient animals and in human cases of beriberi. As a result of a long series of investigations, Peters and collaborators [199] recognized that the oxidation of pyruvate in pigeon brain and other tissues is dependent on the presence of vitamin B_1. More recently Lohmann and Schuster [200] discovered that vitamin B_1 (as the diphosphate) does in fact participate in the oxidation of pyruvate, its rôle being that of cocarboxylase (coenzyme for carboxylase).

$$\text{Lactate} \rightarrow \text{pyruvate} \rightarrow \text{oxidation products}$$
$$\uparrow$$
$$\text{vitamin } B_1\text{-diphosphate}$$

Oxidation of the pyruvate is apparently direct and does not seem to involve the intermediate formation of succinic, α-ketoglutaric, or acetoacetic acid (McGowan and Peters).[199] The suggestion has been made that the neurological manifestations of avitaminosis B in the pigeon are specifically related to the accumulation of pyruvic acid in the brain, owing to failure of its oxidation.

Standardization of Vitamin B_1. The following biological methods have been found useful in the standardization of the antineuritic vitamin (Coward [201]):

1. Cure of retracted neck in avitaminotic pigeons.
2. Cure of paralysis (convulsions) in rats.

[198] A. N. Drury, L. J. Harris, and C. Maudsley, *Biochem., J.*, **24**, 1632 (1930).

[198a] M. Strauss has recently reviewed the use of vitamin B_1 in the treatment of polyneuritis and cardiovascular conditions. *J. Am. Med. Assoc.*, **110**, 953 (1938).

[199] R. A. Peters, *Biochem. J.*, **30**, 2206 (1936); G. K. McGowan and R. A. Peters, *ibid.*, **31**, 1637 (1937); Peters, *Lancet*, **1**, 1161 (1936); see also W. C. Sherman and C. A. Elvehjem, *Am. J. Physiol.*, **117**, 142 (1936); *Biochem. J.*, **30**, 785 (1936), and H. A. Krebs, *Nature*, **138**, 27 (1936).

[200] Naturwissenschaften, **25**, 26 (1937); *Biochem. Z.*, **294**, 188 (1937).

[201] *Nutrition Abstracts & Revs.*, **4**, 705 (1935).

3. Cure of bradycardia, or lowered heart rate, in rats.
4. Increase in weight of rats.

A number of chemical procedures have been proposed for the assay of vitamin B_1, but their specificity has not been fully established. Peters and colleagues [202] have employed the so-called "catatorulin" test, which is based on the effect of the vitamin in elevating the oxygen uptake of brain obtained from vitamin B deficient pigeons (and rats).

Sherman and Chase [203] defined their unit of vitamin B activity on the basis of gain in weight of rats previously depleted of their vitamin B reserve. The Sherman-Chase unit is represented by the amount which fed daily to standard test rats produces a gain of 3 grams per week during a test period of 4 weeks.

The international standard is at present an active fuller's earth adsorbate of the active principle extracted from rice polishings by Jansen and Donath according to the method of Seidell. It will probably be replaced by the pure crystalline vitamin. One international unit is equivalent to 10 mg. of the adsorbate. It corresponds in activity to about 2 Sherman-Chase units. Its equivalence in terms of the crystalline vitamin has not been definitely fixed. According to some estimates, 1 international unit is equivalent to 4–5 γ of the crystalline vitamin, as the hydrochloride. Kinnersley and Peters,[202] using the catatorulin method, found 1 international unit to be equivalent to 2 γ of vitamin B hydrochloride. Harris and Leong [204] by means of their "bradycardia method" estimated that 1 international unit is equivalent to about 4 γ of the vitamin hydrochloride.

Distribution of Vitamin B (B_1) in Foods. Brewer's yeast is the richest known source of vitamin B. Taking it as the standard and assigning the value 100 as the measure of its potency, Chick and Roscoe [205] have compared the vitamin B (B_1) content of various foodstuffs, with the following results, on a dry-weight basis: wheat germ 50; liver (ox), watercress, lettuce, orange, cabbage (etiolated), carrot, spinach, 10–20; green cabbage, turnip, tomato, egg yolk, onion, 5–10; meat (beef), banana, potato, apple, 2.5–5. Owing to differences in water content, the order for these foodstuffs in the fresh form is different. On this basis, wheat embryo is an even richer source than yeast; liver and yolk are excellent, but none of the other substances contain more than one-tenth the vitamin present in yeast and in most the concentration is less.

[202] H. W. Kinnersley and R. A. Peters, *Biochem. J.*, **30**, 985 (1936).
[203] *J. Am. Chem. Soc.*, **53**, 3506 (1931).
[204] *Lancet*, **1**, 886 (1936); see also E. M. Knott, *J. Nutrition*, **12**, 597 (1936).
[205] H. Chick and M. H. Roscoe, *Biochem. J.*, **23**, 498 (1929); *ibid.*, **24**, 1754 (1930); *ibid.*, **25**, 1205, 2050 (1931). See also R. H. A. Plimmer, W. A. Raymond, and J. Lowndes, *Biochem. J.*, **25**, 691, 1788 (1931). The distribution of vitamin B is well summarized by B. Sure, "The Vitamins in Health and Disease," Baltimore, 1933. See also B. L. Oser, in Hawk and Bergheim's "Practical Physiological Chemistry," 11th edition, 1937, Chapter XXXV.

This emphasizes the important point that although vegetables and such fruits as the orange may contribute somewhat to the vitamin B requirement, they cannot be depended upon to furnish very much and hence richer sources must be provided. Not included in the preceding list are nuts, rich in vitamin B. Whole grain cereals are excellent sources. Oysters contain a fair amount of this vitamin; clams do not. Milk, it is now realized, is deficient, as is white bread. Vegetable oils and fats are apparently devoid of vitamin B. With the exception of cream, this seems to be true also of animal fats. Beer as a source of vitamin B is insignificant.

Below are listed a number of common foods and their vitamin B values per 100 grams in international units.

Asparagus	100–135	Onions	10–40
Barley	50–100	Peanuts	300
Beans, dry kidney	125	Potatoes	15–40
Beef	50	Rice, brown	50–100
Cabbage, green	20–35	Rice, white	<20
Cheese, Cheddar	0	Rye	50–75
Cod-liver oil	0	Soybean	200–400
Corn	50–70	Wheat, whole	75–250
Egg white	0	Wheat flour, white	5
Egg yolk	60–125	Yeast, dry, baker's	130–450
Milk, whole	10–25	Yeast, dry, brewer's	1200–6000

Vitamin B_1 Requirement of Man. It has been estimated that an intake of 200–300 international units of the antineuritic vitamin would certainly protect the average adult from beriberi. According to some writers 50–100 units suffices for infants and children, but in all probability a much more liberal allowance is indicated. Children of 3–6 years on a food intake of 2000 calories should have about 300 units of vitamin B. Older children consuming more food probably require up to 400 or 500 units. During pregnancy and lactation the vitamin intake should be 500 units, or more. Cowgill [206] has devoted a monograph to the subject and has discussed at length the relation of the vitamin B requirement to body weight, caloric intake, and other factors. Harris and Leong [204] have determined that 5–8 per cent of the daily vitamin B intake is excreted in the urine.

The capacity of the organism to store vitamin B is very limited, and such reserves as may be present are quickly depleted when the supply in the diet is cut off. Little is known of the effects of excessive amounts of vitamin B, but Perla's [207] experimental studies indicate that the administration of generous amounts is probably without danger. However, in the rat, excessive amounts seem to interfere with reproduction and lactation, especially in the second generation. It is a curious observa-

[206] G. R. Cowgill, "The Vitamin B Requirement of Man," Yale University Press, 1934.

[207] *Proc. Soc. Exptl. Biol. Med.*, **37**, 169 (1937).

tion that some of the effects of hypervitaminosis B (cannibalism, loss of nursing instinct) are the same as those encountered in vitamin B deficiency.

Riboflavin (Lactoflavin Vitamin B_2, or Vitamin G). We have seen that vitamin B was originally thought to be a single substance, but that as a result of certain observations it became apparent that there were at least two distinct principles, one being more heat-stable than the other. The more heat-labile factor was recognized as the antineuritic vitamin (B_1). The heat-stable factor came to be known as vitamin B_2 and also as vitamin G. Autoclaved yeast proved to be an excellent source of the latter. Although it did not alleviate the symptoms of polyneuritis, it proved to be essential for growth. It was also effective in preventing and, in many cases, curing pellagra in man and the pellagra-like disease, "blacktongue," experimentally produced in dogs. A supposedly analogous condition in rats was, likewise, amenable to treatment with yeast.

Vitamin B_2 was for many years looked upon as a single substance, although from time to time some investigators expressed doubt in the matter. In time it was discovered that the vitamin B_2 fraction contained a pigment, which occurred also in milk (lactoflavin), egg white (ovoflavin), liver (hepatoflavin), and elsewhere. Because it contains ribose, it has also been designated as riboflavin. This substance, which we have encountered in another connection (p. 135), proved to be essential for growth in rats. Indeed, the method which Bourquin and Sherman [208] had used in the biological assay of vitamin G (B_2) in foods proved to be almost as useful in estimating this newly recognized flavin component; hence many writers have retained the designation G for this factor.

From the outset it was realized that vitamin G, that is lactoflavin, or riboflavin, did not produce the full and diverse effect of the vitamin B_2 fraction, and accordingly an exhaustive search for other components was initiated in a number of laboratories. It was seen that the pellagra-like dermatitis of the rat was not cured by lactoflavin, and P. György [209] succeeded in showing that, besides lactoflavin, B_2 contained a "rat pellagra preventing" factor. This has been designated as vitamin B_6. However, vitamin B_6 turned out to be ineffective in the pellagra-like condition of dogs and monkeys and in human pellagra, which made it seem probable that autoclaved yeast contained not only riboflavin and vitamin B_6, but also a third factor (Harris [210]). This has been tentatively named the P–P factor, a term originally employed by Goldberger [211] in describing the antipellagra factor of autoclaved yeast.

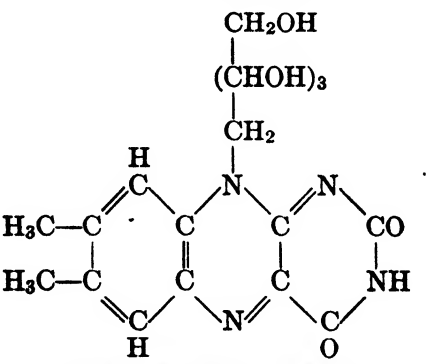
[208] *J. Am. Chem. Soc.*, **53**, 3501 (1931).
[209] *Biochem. J.*, **29**, 741 (1935).
[210] *Ibid.*, **29**, 776 (1935).
[211] J. Goldberger, G. A. Wheeler, *et al.*, U. S. P. H. Reports, **40**, 927 (1925); **41**, 297, 1025 (1926); **42**, 2383 (1927); **43**, 172 (1928).

In a recent study of the different components of "vitamin B_2" Harris [213] has concluded that the monkey, dog, and human being are unable to survive on a basal diet deficient in the human antipellagra, or canine anti-blacktongue, factor (vitamin B_1 and B_6 and lactoflavin (B_2) provided). He has summarized present knowledge of the nutritional importances of these principles for different animals and for man, as shown in the following table. A plus sign indicates that the constituent is needed; a negative sign that it is non-essential; and a question mark that the needs are either doubtful or unknown.

NEEDS OF DIFFERENT SPECIES FOR COMPONENTS OF VITAMIN B_2 COMPLEX

	Lactoflavin	Vitamin B_6	P-P factor
Rats	+	+	−
Dogs	+	+	+
Human beings	?	?	+
Monkeys	?	?	+
Pigeons	?	?	?

Chemistry of Riboflavin (Vitamin G, or lactoflavin). This compound was first isolated in crystalline form from milk by Kuhn, György, and Wagner-Jauregg [213] and subsequently synthesized.[214] As described elsewhere, this substance is the prosthetic group of the "yellow enzyme."



Riboflavin (lactoflavin) or vitamin G

Riboflavin crystallizes as orange yellow needles, melting at 280° C. It is slightly soluble in water and in alcohol, is relatively heat stable, but is destroyed or inactivated on prolonged exposure to visible light.

Nutritional and Physiological Significance. For the rat, vitamin G (riboflavin) is essential for growth. From the work of Day [215] and collab-

[213] *Biochem. J.*, **31**, 1414 (1937).

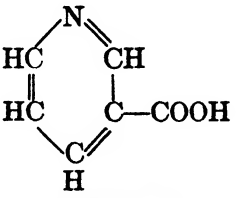
[213] *Ber.*, **66B**, 1034 (1933).

[214] P. Karrer, *et al.*, *Helv. Chim. Acta*, **18**, 426 (1935); R. Kuhn *et al.*, Naturwissenschaften, **23**, 260 (1935).

[215] *Am. J. Ophthalmol.*, **14**, 1005 (1931); P. L. Day and W. J. Darby, *J. Nutrition*, **12**, 387 (1936); **13**, 389 (1937). The chemical aspects of riboflavin have been reviewed recently by L. E. Booher, *J. Am. Med. Assoc.*, **110**, 1105 (1938).

orators it seems that deficiency of the flavin factor may predispose to cataract formation in rats. The relation of the vitamin to the "yellow enzyme" (p. 135) makes it very probable that it is of fundamental importance physiologically, but apart from this extraordinary fact, little is known at present concerning the rôle of this vitamin in *human* nutrition.

The P-P Factor and Other Components. Goldberger [216] designated the pellagra-preventing principle of autoclaved yeast as the P-P factor. Elvehjem and collaborators [216] have recently reported the isolation of nicotinic acid amide from liver concentrate and have also discovered that it cured dogs of "blacktongue" induced by feeding a pellagra producing diet. This report was followed within a remarkably short time by several announcements that nicotinic acid was equally effective as a therapeutic agent in human pellagra.[217]



Nicotinic acid
(M-pyridine-carboxylic acid)

It is not improbable therefore that nicotinic acid is either the antipellagra factor or a component of the P-P vitamin. Of considerable interest is the fact that three of the known components of the vitamin B complex (B_1, riboflavin and nicotinic acid) play essential rôles in reactions of cellular oxidation. In passing it should be recalled that Funk,[140] in his pioneer work on vitamin B, discovered the presence of nicotinic acid in extracts of rice polishings and yeast.

Vitamin B_6 (also called vitamin H and factor Y), as we have seen, is the antidermatitis factor in the rat. Nothing is known of its rôle in human nutrition and little can be said at present concerning its chemical properties.

Even more intangible are factors B_3, B_4 and B_5. Vitamin B_3 (heat labile) is believed to be essential for growth in the pigeon. B_4 is supposedly a specific antiparalytic factor for rats and chicks. Evidence recently submitted however indicates that so-called "vitamin B_4 deficiency" may be due to partial deficiency of vitamin B_1 and may be cor-

[216] C. A. Elvehjem, R. J. Madden, F. M. Strong, and D. W. Woolley, *J. Am. Chem. Soc.*, **59**, 1767 (1937); see also H. R. Street and G. R. Cowgill, *Proc. Soc. Exptl. Biol. Med.*, **37**, 547 (1937); H. Chick *et al.*, *Biochem. J.*, **32**, 10 (1938).

[217] P. J. Fouts, O. M. Helmer, S. Lepkovsky and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.*, **37**, 405 (1937); D. T. Smith, J. M. Ruffin, and S. G. Smith, *J. Am. Med. Assoc.*, **109**, 2054 (1937); T. D. Spies, C. Cooper, M. A. Blankenhorn, *ibid.*, **110**, 622 (1938).

rected by increasing the intake of the latter. Vitamin B_5 (heat stable) is thought to be a weight maintenance factor for the pigeon. There have also been described two additional principles: factor *W*, a growth essential for the rat, and the so-called filtrate factor required for the prevention of a nutritional dermatosis in chicks.

The better known components of the vitamin B complex may therefore be summarized as follows:[218]

	Function	Chemical Constitution
Vitamin B_1.....	Prevents beriberi in man and polyneuritis in animals.....	p. 585
Riboflavin.....	(a) Component of an oxidation-reduction system of living cells. (b) Prevents cataracts in rats. (c) Necessary for growth in chicks and rats.	p. 592
P-P factor.....	Prevents and cures pellagra in man and a similar condition in monkeys, as well as "blacktongue" in the dog.....	Nicotinic acid?
Vitamin B_6	Antidermatitis factor in rat.....	Unknown

Vitamin C, or Ascorbic Acid, The Antiscorbutic Vitamin

Authentic accounts of scurvy may be found in the literature of the fifteenth century. Lind in 1752 wrote a treatise on scurvy in which the specific value of oranges, lemons, citrons, and apples as antiscorbutics is clearly stated. This is indeed remarkable, for we rarely go back so far to past generations for authoritative accounts of nutritional disorders. The history of scurvy has been traced by Hess [219] in his monograph on the subject and more briefly by Harris. [220]

As to the prevalence of scurvy, it is stated that between 1556 and 1887 there are known to have occurred 143 "epidemics" of this disease on land. Most of the outbreaks were among troops, prisoners, and inmates of institutions. In times of famine and war, scurvy has been very prevalent. During the siege of Paris, which lasted from September 17, 1870, to January 27, 1871, scurvy broke out among the inmates of the prisons, patients in the military hospitals, as well as among the civilian population. In the Russo-Japanese War, after the siege of Port Arthur, it was found that one-half of the garrison of 17,000 men had scurvy. Scurvy was likewise prevalent during the Great War, the highest incidence having been reported from Austria. Both the civilian population and troops suffered from this deficiency disease in Russia. It is at present

[218] For a recent summary of the components of the vitamin B complex and references to the literature the reader is referred to E. M. Nelson, *J. Am. Med. Assoc.*, **110**, 645 (1938).

[219] A. F. Hess, "Scurvy—Past and Present," Lippincott, Philadelphia, 1920; see also *J. Am. Med. Assoc.*, **98**, 1429 (1932).

[220] L. J. Harris, "Vitamins in Theory and Practice," Macmillan, 2d edition, 1937.

very common among the natives of South Africa and the aborigines of Central Australia.

Scurvy has been more frequently associated with life at sea. On long voyages, such as those made by the early explorers, serious outbreaks of the disease occurred. An early account of such an outbreak is that of Vasco da Gama, who, about 1497, reached the East Indies by way of the Cape of Good Hope. Of a crew of 160 men, about 100 perished. Harris tells of a Spanish galleon that was found adrift at sea, its entire crew dead from scurvy. In our own day, explorers have suffered most from scurvy, particularly in Arctic and Antarctic expeditions. This does not apply, of course, to the well-organized expeditions of the last few years, such as those of Rear Admiral Byrd. The relation of diet to scurvy has been known to sailors and explorers since the eighteenth century. Sprouted barley, sauerkraut, beans, lentils, greens, grass, and decoctions from the needle of the Spruce and pine were once used as a protection against the disease. The high incidence of scurvy at sea is readily comprehended when one considers that for many months the diet of the sailors lacked fresh animal and vegetable foods. The efficacy of the juice of limes, lemons, and oranges was likewise known at an early date, and, in 1795, lime and lemon juice were introduced into the rations of sailors in the British Navy, with the result that the incidence of scurvy decreased to a remarkable extent. Occasionally outbreaks occurred on what appeared to be liberal allowances of lime juice and consequently many lost faith in its value as an antiscorbutic. As we shall see presently, the antiscorbutic substance is readily destroyed, and this may have occurred in the process of preparation and preservation of the juice.

The more prominent symptoms of scurvy in man are loss of weight, pallor (due often to anemia), weakness, breathlessness, palpitation of the heart, swelling of the gums, loosening of the teeth, hemorrhage into the skin and mucous membranes, pains in the bones and joints, edema, nervousness, and hypersensitivity to pain. Scorbutic patients frequently die in delirium, but death may also occur suddenly, suggesting the possibility of cardiac involvement.

In 1895, Theobald Smith [221] reported a peculiar hemorrhagic condition in guinea pigs restricted to a diet containing cereal but no grass, clover, or succulent vegetable like cabbage. The importance of this observation was not appreciated until Holst and Frölich,[222] in 1912, pointed out the similarity between this disease in guinea pig and human scurvy. The resemblance is indeed very striking, for a diet that causes scurvy in man likewise produces it in the guinea pig, and those substances that exert a curative effect in man are equally efficacious in experimental scurvy in the guinea pig. However, despite the acceptance of the vitamin theory in relation to beriberi and xerophthalmia, the

[221] U. S. Dept. Agr. Bureau Animal Industry Ann. Rept., 1895–96, 172.
[222] *Z. Hyg. Infect.-Krank.*, **72**, 1 (1912).

idea that scurvy might also be a deficiency disease, brought about by some form of avitaminosis, did not gain much headway at first. It required the convincing observations of Chick and Hume,[223] Cohen and Mendel,[224] Harden and Zilva [225] and others before it was finally accepted that scurvy was caused by a deficiency of a specific factor. In 1919 Drummond [226] designated the antiscorbutic substance as vitamin C.

The work which followed had many objectives, such as the development of methods of standardization and assay, determination of distribution in plant and animal tissues, the requirements and rôle in nutrition, the significance of the vitamin in certain physiological processes, the pathological changes resulting from deficiency, and finally the matter of its isolation, identification, and synthesis. And, as we shall see, even the last achievements did not close the problem of scurvy, but rather opened new paths and revealed new horizons for further investigation. We shall first consider briefly some of the chemical aspects of vitamin C.

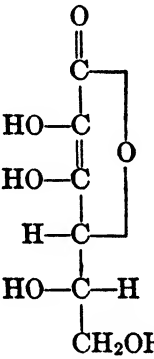
Chemical Constitution and Properties of Vitamin C. In 1928 Szent-Györgyi [227] isolated a reducing substance from various plant sources (orange, lemon, cabbage, etc.) and from the suprarenal cortex. He recognized it to be *hexuronic acid*, $C_6H_8O_6$. As a result of a series of investigations by Tillmans and Hirsch [228] a remarkable correlation was disclosed between the reducing potential of various foods and their vitamin C content. Working independently, King and associates [229] in preparing vitamin C concentrates from lemon juice were impressed by the strong reducing action of the physiologically active material, and in 1932 Waugh and King [230] finally succeeded in isolating a crystalline substance which on repeated recrystallization remained constant in its antiscorbutic activity, 0.5 mg. daily being adequate to protect the guinea pig against scurvy. This crystalline material proved to be identical with the hexuronic acid first isolated by Szent-Györgyi [227] (and later by Kendall)[231] from the adrenals and other sources.

At about the same time, Svirbely and Szent-Györgyi [232] also recognized the antiscorbutic property of their hexuronic acid. Pursuing the problem further [233] and employing the red pepper (*Capsicum annum*) as a

[223] *J. Roy. Army Med. Corps.*, **29**, 121 (1917); *Proc. Roy. Soc.* (*London*), *B*, **90**, 44 (1917).
[224] *J. Biol. Chem.*, **35**, 425 (1918).
[225] *Biochem. J.*, **12**, 408 (1918).
[226] *Ibid.*, **13**, 77, (1919).
[227] *Biochem. J.*, **22**, 1387 (1928).
[228] *Untersuch. Lebensm.*, **60**, 34 (1930); **63**, 1, 27, 241, 267, 276 (1932); *Biochem. Z.*, **250**, 312 (1932).
[229] J. L. Svirbely and C. G. King, *J. Biol. Chem.*, **94**, 483 (1931–32); F. L. Smith and King, *ibid.*, **94**, 491 (1931–32).
[230] *Ibid.*, **97**, 325 (1932).
[231] *Proc. Staff Meetings Mayo Clinic*, **6**, 296 (1931).
[232] *Biochem. J.*, **26**, 865 (1932).
[233] *Ibid.*, **27**, 279 (1933).

source they obtained 450 grams of the acid in crystalline form. To prove its identity with vitamin C they prepared the monoacetone derivative, recrystallized it, and then recovered the hexuronic acid. The final product possessed the same activity as the original material, and in agreement with the observations in King's laboratory, 0.5 mg. was found to be the protective daily antiscorbutic dose for the guinea pig. The name, *ascorbic acid*, was proposed by Haworth and Szent-Györgyi.[234]

In the meanwhile, investigators in several laboratories were actively engaged in the attempt to determine the chemical constitution of this substance. The first report that this end had been achieved came from Haworth's laboratory (Herbert, Hirst, *et al.*[235]), to be followed soon after by confirmatory reports from other workers. Ascorbic acid may be represented by the following structural formula:



l-Ascorbic acid or vitamin C
(2,3-dienol-*l*-gulonic acid lactone)

It is worthy of note that the synthesis of the vitamin was accomplished by Reichstein, Grüssner, and Oppenhauer,[236] in Karrer's laboratory, before its constitution was definitely established. At first they prepared from *d*-xylose the isomeric *d*-form, which is physiologically inert, but followed this immediately by the synthesis of the natural *l*-form from *l*-xylose. The synthesis included the following sequence of transformations:

l-Xylose → xylosazone → xylosone → xylosone nitrile → *l*-ascorbic acid

In Haworth's laboratory, *d*-galactose was used as the starting material; this was converted through a series of reactions into *l*-xylose and eventually into *l*-ascorbic acid. A number of other procedures have been developed; one of these (Reichstein and Grüssner[237]) has found application in the commercial synthesis of the vitamin. *d*-Glucose is

[234] *Nature*, **131**, 24 (1933).

[235] R. W. Herbert, E. L. Hirst and associates, *J. Chem. Soc.* (*London*), 1270, 1419, 1564 (1933); W. N. Haworth, *et al.*, *J. Soc. Chem. Ind.*, **52**, 221, 482 (1933).

[236] *Nature*, **132**, 280 (1933); *Helv. Chim. Acta*, **16**, 561, 1019 (1933).

[237] *Helv. Chim. Acta*, **17**, 311 (1934); **18**, 608 (1935).

first reduced to *l*-sorbitol by hydrogen and palladium. The succeeding steps may be indicated as follows:

$$l\text{-sorbitol} \xrightarrow{\textit{Bact. xylinum}} l\text{-sorbose} \xrightarrow{\text{Acetone}} \text{diacetone sorbose} \xrightarrow{KMnO_4} \text{diacetone sorburonic acid} \longrightarrow$$

$$l\text{-sorburonic acid (2-keto-}l\text{-gulonic acid)} \rightarrow l\text{-ascorbic acid}$$

A method somewhat similar to this was developed independently in King's laboratory.[238]

As has been mentioned, natural ascorbic acid is the *l*-variety. The synthetic compound is identical with it in every respect, both chemically and physiologically. It is a white, crystalline substance soluble in water, methyl alcohol, ethyl alcohol, and acetone, but not in other fat solvents. It melts sharply at 192° C.; specific rotation in water +24°; no mutarotation; maximum absorption is at 260 mμ in water solution. It is strongly reducing and unstable toward alkali and oxidizing agents, I_2, NaIO, $KMnO_4$, etc.; it is relatively stable in alkaline solution in the absence of oxygen.

Ascorbic acid reduces the indicator 2:6 dichlorophenolindophenol (indophenol dye). The vitamin is thereby oxidized to dehydroascorbic acid.[239] The reaction may be represented as follows:

$$\underset{\text{Ascorbic acid}}{C_6H_8O_6} + O{:}\,\underset{\text{Indophenol dye}}{C_6H_2Cl_2{:}\,N\cdot C_6O_4OH} \rightarrow \underset{\text{Dehydroascorbic acid}}{C_6H_6O_6} + \underset{\text{Leucobase (colorless)}}{HO\cdot C_6H_2Cl_2\cdot NH\cdot C_6H_4OH}$$

This property has been utilized in the development of titration methods for the quantitative estimation of the vitamin C content of foods, urine, blood, tissues, etc.[240]

Long before the chemical nature of vitamin C was even suspected it was known to be readily destroyed by heat, especially in the presence of oxygen. For example, cabbage when boiled for one hour at a temperature of about 100° C. may lose as much as 90 per cent of its antiscorbutic activity. Heating at high temperatures for short periods is less destructive than heating at lower temperatures for longer periods. The destruction of the vitamin is essentially an oxidative process.

[238] *Physiol. Rev.*, **16**, 238 (1936).

[239] The structural formula of dehydroascorbic acid may be written as follows: $CH_2OH\cdot CHOH\cdot \underset{\vert\!\!-\!\!O\!\!-\!\!\vert}{CH}\cdot CO\cdot CO\cdot CO$. This is the reversibly oxidized form of ascorbic acid and may be reconverted into the vitamin. Further oxidation converts dehydroascorbic acid into a more strongly reducing substance, which cannot be reconverted into the original ascorbic acid. See Borsook, *et al.*, *J. Biol. Chem.*, **117**, 237 (1937); Barron, *et al.*, *ibid.*, **116**, 563 (1936).

[240] The procedure was originally developed by J. Tillmans, P. Hirsch, and colleagues: *Z. Untersuch. Lebensm.*, **63**, 1, 276 (1932); **65**, 145 (1935); *Biochem. Z.*, **250**, 312 (1932). Improvements and modifications have been introduced by L. J. Harris and S. N. Ray, *Biochem. J.*, **27**, 303, 2006 (1933); see also *ibid.*, **27**, 590 (1933), and O. A. Bessey and C. G. King, *J. Biol. Chem.*, **103**, 687 (1933).

Vitamin C is likewise destroyed in the process of drying and aging of foods. Foods may also lose their antiscorbutic property during storage. Evidently the presence of copper catalyzes the oxidative destruction of vitamin C. These factors have constituted serious problems in the preserving, canning, and storage of food. Accordingly, procedures have been developed, especially in the canning industry, with the object of minimizing oxidation during the heating process.

Nutritional Importance of Vitamin C. Much of our knowledge of vitamin C deficiency we owe to the fact that scurvy in the guinea pig is essentially the same disease as it is in man and monkeys. It is also a remarkable fact that certain animals—rat, mouse, prairie dog, dog, pigeon, domestic fowl, cow, etc.—do not require vitamin C. These animals are evidently capable of synthesizing it, for even when kept on a scorbutic diet, the vitamin is found in their tissues.

Among the conspicuous manifestations of scurvy (see also p. 595) are diminished capillary resistance, marked tendency to hemorrhage, structural changes in cartilage, bone, and teeth, and enlargement of the adrenals. According to Wolbach and Howe,[241] the fundamental defect is the loss of ability of the supporting tissue to produce and maintain intercellular substances. As these cementing and supporting materials are resorbed and nothing is provided to take their place, there being no new formation of intercellular material, reparative processes cease. As there is a lack of fibrous and collagenous substance, structural weakness results. This is general, affecting endothelium, cartilage, bone, etc. As a result of these morphological changes, hemorrhage follows even slight trauma, disunion of the epiphysis from the diaphysis tends to occur in children; there is separation of periosteum from bone, as well as fracture and fragmentation of bone. Deficiency in dentine formation and of intercementing material is associated with the separation of the remaining dentine from the pulp.

The specificity of vitamin C in relation to these changes is proved by the promptness and extent of the reparative processes which follow the institution of vitamin C therapy (natural food sources, as well as ascorbic (cevitamic acid). Wolbach states that the histologic repair is dramatic; newly formed dentine, collagen, and bone matrix can be seen after twenty-four hours. There is new capillary formation; blood clots show beginning organization, and bone fractures show signs of early repair.[242]

Apart from the general effects of good nutrition on resistance to disease, there is a certain amount of evidence relating vitamin C directly to resistance against certain infections. For example, King and Menten [243] observed that the resistance of guinea pigs to diphtheria toxin is improved by allowing a liberal intake of vitamin C. It has also been

[241] *Arch. Pathol.*, **1**, 1 (1926); *J. Am. Med. Assoc.*, **108**, 7 (1937).

[242] For fuller discussions the reader is referred to the review by C. G. King, *Physiol. Rev.*, **16**, 238 (1936), and to the one by G. Bourne, *ibid.*, **16**, 442 (1936).

[243] *J. Nutrition*, **10**, 129 (1935).

noted that experimental tuberculosis in the guinea pigs runs a severer course in animals with chronic vitamin C deficiency than in those generously provided in this respect.

Compared to its former incidence, scurvy is now comparatively rare, though it is still encountered, not infrequently, among individuals of all age groups. There is, however, a growing conviction that a very much larger number probably suffers from latent, or subclinical, scurvy. This is usually revealed by symptoms similar to those seen in scurvy, but in a milder form (sore or bleeding gums, painful joints, reduced capillary resistance,[244] etc.). Another criterion has recently received attention. It is an outgrowth of the view put forward by Johnson and Zilva [245] that the body tissues can store vitamin C, but only up to a certain point. When the "saturation state" is reached no more vitamin is stored; all that is subsequently ingested is either utilized in some other way or excreted. Accordingly, an individual with a maximum reserve excretes a larger proportion of the vitamin taken than one who is in a depleted state (subclinical scurvy). The latter stores more of it and excretes relatively less. By determining the amount of vitamin C excreted after the administration of a test dose, an idea may be had of the subject's vitamin C reserve, especially if this test is repeated daily over a period of several days.[246]

From the foregoing it is seen that the organism is capable of storing a limited amount of vitamin C; however, in animals in which this substance is not synthesized, the reserve is rapidly depleted when the supply of the vitamin in the diet is cut off. The highest concentrations of vitamin C occur in the adrenals, corpus luteum, interstitial cells of the testis, and the anterior pituitary. The liver is also a site of storage.

The participation of ascorbic acid in the reactions of cellular oxidation, which has been briefly discussed (p. 304), points to the fundamental importance of the vitamin in physiological processes. The vitamin appears to be a component of certain enzyme systems in which it acts as an hydrogen-transporter between organic metabolites. Considerable interest has been aroused over the property of ascorbic acid in activating such enzymes as arginase, cathespin, and phosphatase.[247]

[244] A clinical method has been introduced to test the ability of small blood vessels to withstand increased intravascular pressure. This is the so-called "capillary resistance test." G. F. Göthlin, *Skand. Arch. Physiol.*, **61**, 225 (1931); *Nature*, **134**, 569 (1934); see also G. Dalldorf, "A Sensitive Test for Subclinical Scurvy in Man," *Am. J. Diseases of Children*, **46**, 794 (1933); *J. Am. Med. Assoc.*, **104**, 1701 (1935).

[245] *Biochem. J.*, **28**, 1393 (1934); L. J. Harris and S. N. Ray, *Lancet*, **1**, 71 (1935).

[246] In scorbutic infants and children the daily excretion of vitamin C in the urine was found to be less than 0.7 mg., compared to 1.1–5.3 mg. in a similar group ill from other diseases. The administration of test doses of 85–170 mg. of the vitamin (as ascorbic acid or orange juice) had practically no effect on the urinary output of the scorbutic subjects, but definitely raised the output (5–10.9 mg.) of those without history of scurvy.

[247] S. J. Thannhauser, M. Reichel and J. F. Grattan, *J. Biol. Chem.*, **121**, 697 (1937).

Standardization and Assay. The international standard is a particular specimen of *l*-ascorbic acid, and the international unit of vitamin C activity is represented by 0.05 mg. of this standard. This unit has replaced the original international unit which was defined as the vitamin C activity of 0.1 gram of freshly expressed lemon juice.

In the biological methods of assay of vitamin C potency, the guinea pig has served as the test animal. The procedure that has proved to be especially useful is the one recommended by Sherman, La Mer, and Campbell.[248] The basal diet consists of measured amounts of ground whole oats, heated skim milk powder, butterfat, table salt, yeast, and cod-liver oil. Such a diet invariably produces scurvy, rapid loss of weight, and death in about four weeks. Protection may be obtained by an allowance of food containing vitamin C. The minimum amount of food which affords complete protection against scurvy in a standard guinea pig is determined. This corresponds to about 10–12 international units of vitamin C (about 0.5 mg. of ascorbic acid). It is to be noted that, in the actual conduct of biological assays of vitamin C, whether by the method of Sherman, or Zilva, or some other method, considerable experience and judgment are required in evaluating the severity of the scorbutic lesions and other results.[248]

Disorganization of tooth structure (especially of the roots of the incisors) in guinea pigs on a scorbutic diet and its prevention by feeding vitamin C containing foods has also been made the basis of a quantitative method of assay.

The most widely used of the chemical procedures for the estimation of vitamin C is the titration method with 2,6-dichlorophenolindophenol. The plant or animal tissue to be analyzed is first extracted with a solution of trichloracetic (or acetic) and metaphosphoric acids and the extract is then titrated with a standard solution of the dye.

Roe [249] has developed a colorimetric method which involves the conversion of the ascorbic acid into furfural, reaction of the latter with aniline under certain specified conditions, and comparison of the resulting colored solution with appropriate standards. Gál [250] has described a titration procedure in which standard methylene blue is added in excess and the portion not reacting with the ascorbic acid is determined by back titration with titanium chloride.

The specificity of these and other chemical procedures has not been fully established.

Fruits, especially of the citrus variety, vegetables and berries are as a rule good or excellent sources of vitamin C. Milk is a relatively poor source of vitamin C.[251] Grain products, sugars, starches, fats, oils, and

[248] For fuller details the reader is referred to H. C. Sherman and S. L. Smith, "The Vitamins," 2d edition, Chemical Catalog Co., New York, 1931.

[249] *J. Biol. Chem.*, **116**, 609 (1936).

[250] *Nature*, **138**, 799 (1936).

[251] It has been estimated that 500 cc. of cow's milk (16 ounces), per day, is the

yeast do not contain this vitamin. Among the common articles of diet in which vitamin C is abundant are: lemon, orange, grapefruit, mango, pineapple, certain varieties of apple (particularly in the skin), tomato, cabbage, parsley, watercress, fresh spinach, rutabaga, leaf lettuce, strawberries. Svirbely and Szent-Györgyi [233] have discovered that paprika (Hungarian red pepper) is very rich in vitamin C. According to the analyses of Bessey and King,[252] red pepper contains 2.3 mg. per gram, green pepper 1.83 mg., as compared with 0.57 mg. for lemon juice, 0.53 mg. for grapefruit, and 0.71 mg. for oranges. Among animal tissues, the suprarenal cortex is conspicuous for its high vitamin C content, as first shown by Szent-Györgyi. The corpus luteum is equally rich. According to the analyses of Bessey and King, these contain 1.4 to 2.3 mg. per gram. The brain, liver, testes, ovaries, and other glandular tissues comprise a second group ranging considerably lower (0.1–0.4 mg. per g.).

Vitamin C Requirement for Man. King [242] states that the human requirement per day ranges from about 25 mg. in infancy to 40 mg. in adults. In pregnancy a more liberal allowance is indicated.

The average adult excretes about 20 mg. of vitamin C daily, even when the diet is moderately low with respect to this substance. Higher values (32–38 mg.) were observed by Ray and Harris [245] in persons whose diet contained liberal amounts of fruits and vegetables. Lower values (11.74 mg.) were observed in a subject who ate little fruit or vegetables.

Is Scurvy Due to a Deficiency of More Than One Vitamin? Vitamin P. The experimental and clinical work which followed the identification of vitamin C as ascorbic acid supported the view that this was in fact the antiscorbutic substance itself. The possibility that scurvy might be due to a lack of vitamin C plus some other substance, or substances, seemed remote, although the following penetrating remark by King [242] in 1936 indicates that the question had not been entirely overlooked: "The number of examples where there is a coincident high concentration of vitamin C and green, yellow and red pigments affords a basis for interesting speculation and future experimentation, but so little is definitely established concerning the rôle of either the pigments or the vitamin that one can scarcely do more than note the association in occurrence at the present time. Many plant physiologists are of the

minimum required to protect an infant from scurvy. Hess's experience has been that 12 ounces of the best grade of raw milk, per day, is at times insufficient to effect a cure. Breast-fed infants and those given raw milk are much less likely to develop scurvy than are infants maintained exclusively on pasteurized, condensed, or evaporated milk. The greatest incidence has been observed in cases where proprietary infant foods have been permitted to replace a part of the daily milk allowance. The analyses of Harris and Ray show that whereas 1 cc. of cow's milk contains an average of .02 mg. of ascorbic acid, 1 cc. of human milk contains an average of .056 mg. The necessity of providing a supplement of vitamin C from such rich sources as orange or tomato juice to nursing infants is now generally recognized.

[252] *J. Biol. Chem.*, **103**, 687 (1933).

opinion that the associated pigments are participants in cellular respiration, and the parallel occurrence of vitamin C in high concentration adds some weight to this point of view."

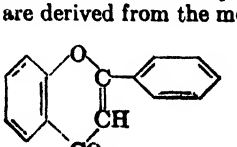
Early the same year, Rusznyák and Szent-Györgyi [253] published a note in which they stated that in certain pathological conditions characterized by increased permeability or fragility of the capillary wall (hemorrhagic purpura, septic conditions, etc.), ascorbic acid is ineffective, while extracts of Hungarian red pepper and lemon juice were definitely beneficial. The extracts were fractionated and the active principle turned out to be in the fraction containing almost pure flavone, or flavonol glucoside,[254] 40 mg. of this fraction proved therapeutically effective in a case of spontaneous bleeding due to decreased capillary resistance. Rusznyák and Szent-Györgyi suggested that the great group of flavones or flavonols play an important rôle in animal life and proposed the name "vitamin P" for the substance responsible for the action on vascular permeability.

The active substance was later obtained in crystalline form (Bentsáth, Rusznyák and Szent-Györgyi [253] and named "citrin." The administration of 1 mg. of this, daily, to guinea pigs kept on a scorbutic diet did not prevent scurvy; however, life was prolonged and the hemorrhages were much less frequent and less severe than in the control animals not receiving "citrin." The authors therefore concluded that experimental scurvy is due to a combined deficiency of vitamins C (ascorbic acid) and P (citrin). Certain of these observations could not be verified; vitamin P according to Zilva [255] had no effect in prolonging the life of vitamin C deficient guinea pigs. To meet these objections, Bentsáth and Szent-Györgyi offered the explanation that vitamin P requires for its activity traces of ascorbic acid and that, in the absence of all trace of vitamin C, its effect can not be fully demonstrated.

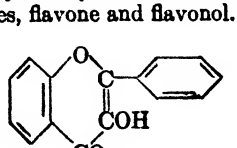
Further work disclosed (Bruckner and Szent-Györgyi) that "citrin" consists of mixed crystals of two related flavone dyes: the glucoside hesperidin, $C_{50}H_{60}O_{27}$ (rhamnose + 2 glucose + hesperitin) and the glucoside of eriodictyol. The latter glucoside is demethylated hesperi-

[253] *Nature*, **138**, 27, 798, 1057 (1936); **139**, 326 (1937); **140**, 426, 588 (1937); *Deutsch. med. Wochschr.*, **62**, 1325 (1936); *Z. physiol. Chem.*, **247**, 258 (1937).

[254] The flavone and flavonol pigments are very widely distributed in the higher plants and are derived from the mother substances, flavone and flavonol.



Flavone



Flavonol

Consult M. W. Onslow, "Practical Plant Biochemistry," Cambridge University Press, 1923, p. 110.

[255] *Biochem. J.*, **31**, 915, 1488 (1937).

din. It is stated that eriodictyol glucoside is formed from hesperidin by demethylation during the ripening of fruit.

Vitamin D

In 1919, Mellanby [256] published a paper in which he described the experimental production of rickets in puppies and set forth the view that rickets is due probably to a lack of the fat-soluble A vitamin. In 1922, McCollum, Simmonds, Becker, and Shipley [257] subjected cod-liver oil to oxidation, thereby destroying vitamin A, as demonstrated by the fact that the product failed to cure xerophthalmia. It was now a question of determining whether the power to cure rickets had likewise been destroyed in the process. A year earlier, Shipley, Park, McCollum, Simmonds, and Parsons [258] were able to show that the sudden introduction of cod-liver oil into the diet of a rachitic rat is followed by a beautiful deposition of lime salts in bone, in a transverse line across the cartilage, at right angles to the long axis of the shaft. This phenomenon was utilized by this group of investigators at Johns Hopkins University as a delicate biological test, the so-called "line test," by which curative effects in rickets could be determined. By means of this method, McCollum and his associates found that the oxidized cod-liver oil, though lacking vitamin A, had nevertheless retained its power of curing rickets. It was determined, moreover, that coconut oil, though deficient in fat-soluble A, possessed the power of stimulating the deposition of calcium in a manner similar to that of cod-liver oil. It was therefore concluded "that the power of certain fats to initiate the healing of rickets depends on the presence in them of a substance which is distinct from fat-soluble A." The existence of a fourth vitamin, or vitamin D, was thus postulated.

In the meanwhile, definite information was obtained concerning the beneficial effect of ultraviolet light, as well as sunshine, in the healing of rickets in children and in experimental animals. The value of sunlight in the treatment of this disease had been stressed for a long time, but the actual demonstration that it promoted the deposition of calcium in bone was of necessity delayed until the X-ray made periodic examination of the condition of the bones a possibility. It was determined that light of wavelength 300 mμ, or shorter, was most effective. Since ordinary window glass shuts out these rays, the light received by a child behind windows does not protect it against rickets.

The problem of rickets took an unexpected turn with the appearance, in 1924 and 1925, of a number of papers in which was described the remarkable effect of ultraviolet irradiation in endowing foods, otherwise ineffective, with antirachitic potency. Attention was called to this phenomenon at approximately the same time by two groups of workers in this country, Hess and Weinstock, and Steenbock and his students.

[256] *Lancet*, **1**, 407 (1919). [257] *J. Biol. Chem.*, **53**, 293 (1922).
[258] *Ibid.*, **45**, 343 (1921).

It was observed by Steenbock and Nelson [259] that a ration which ordinarily induced rickets in rats could be made definitely antirachitic by the simple expedient of exposing it to ultraviolet light. Hess [259a] reported that, by means of irradiation, vegetable oils, green vegetables, dry milk, and other substances acquired antirachitic properties.

Bills, [260] in reviewing the earlier development of knowledge of vitamin D (1922–1932), enumerates five particular discoveries which stood out from among the painstaking investigations upon which they were based. The first of these we have mentioned; it was the identification of vitamin D as a substance distinct from vitamin A (McCollum and associates, 1922). The second has also been stated; it was the discovery that by ultraviolet irradiation foodstuffs could be endowed with the antirachitic property exhibited by fish oils (Steenbock and associates; Hess and associates, 1924). It was shown, moreover, that activation of the foodstuffs was produced by the same short wavelengths as were effective directly in the cure of rickets. This was followed almost immediately by the discovery that the acquisition of antirachitic potency by foods and other materials was due to the activation of the sterol fraction.[261]

The two discoveries which followed, namely, the "identification" of ergosterol as the parent substance of vitamin D (1927),[262] and the isolation of calciferol (p. 607), the active component of irradiated ergosterol (1931–33), will be summarized, along with subsequent developments, in the following paragraphs on the chemical properties of vitamin D.

Chemical Constitution and Properties of Vitamin D. Part of the evidence which in 1925 led to the conclusion that the antirachitic activity of fish oils and of irradiated foods resided in the sterol fraction was based on the demonstration that ordinary cholesterol could be activated by irradiation. It soon became evident, however, from spectrographic [263] and other data,[262] that the precursor of vitamin D was not cholesterol, but some other sterol associated with it. Attention was almost at once directed to ergosterol (p. 607) as the possible provitamin, because this unsaturated sterol was found to exhibit the same four spectral absorption bands as characterized the unsaponifiable fraction of oils and other materials, which became antirachitic on irradiation, and also because ergosterol and the unknown provitamin were alike destroyed by certain oxidizing agents.

[259] *J. Biol. Chem.*, **62**, 209 (1924–25).

[259a] *Am. J. Diseases of Children*, **28**, 517 (1924); *J. Biol. Chem.*, **62**, 301 (1924–25); **64**, 181 (1925).

[260] C. E. Bills, "The Multiple Nature of Vitamin D," *Cold Springs Harbor Symposia on Quantitative Biology*, **3**, 328 (1935).

[261] Hess and Weinstock, *J. Biol. Chem.*, **64**, 181, 193 (1925); Steenbock and Black, *ibid.*, **64**, 263 (1925).

[262] Rosenheim and Webster, *Biochem. J.*, **21**, 127, 389 (1927); Windaus and Hess, *Nachr. ges. Wiss., Göttingen*, **175**, 84 (1927).

[263] F. W. Schlutz and M. Morse, *Am. J. Diseases of Children*, **30**, 199 (1925); R. Pohl, *Nachr. ges. Wissensch. Göttingen, Math.-physik. Klasse*, 142 (1926); cited by Bills.

Now it should be made clear that considerable difficulty was encountered by several investigators [264] in purifying cholesterol so that it would lack the activating constituent. Repeated recrystallization, though it yielded very pure cholesterol, did not entirely remove the provitamin. However, in 1927 methods were described by two groups of investigators (Rosenheim and Webster; Windaus and Hess [262]) by which cholesterol was said to be separated from the potentially active sterol. One was by means of purifying charcoal; the other by bromination and debromination. The latter process supposedly destroyed the provitamin, and since ergosterol is completely destroyed by bromine, it seemed logical to conclude that this was probably the substance concerned.

Certain workers, notably Bills, Steenbock, Koch, and their associates,[264] were unable to confirm these results. They found that cholesterol purified with charcoal, or by repeated treatment with bromine and recrystallization, was still capable of being activated. However, at the time, the weight of evidence seemed to be in the other direction, especially as it was definitely proved that ergosterol, which is itself inactive, acquired the properties of vitamin D upon being exposed to ultraviolet light.

Efforts directed toward the isolation of the vitamin finally met with success. In 1930–31, Askew and co-workers [265] separated from among the irradiation products of ergosterol a crystalline substance which they considered to be the active principle. It was designated *calciferol.* At about the same time, Windaus and associates, [266] using a different method, likewise succeeded in isolating the vitamin, which they described as vitamin D_1, inasmuch as there were indications that a second antirachitic principle existed. Further research disclosed, however, that D_1 was in reality the vitamin in combination with an inert isomer, *lumisterol.* Askew found that his original calciferol was likewise not the pure vitamin, but that it was combined with two biologically inactive sterols, one of these being lumisterol. Further purification resulted in the isolation of the vitamin; this was accomplished independently in the laboratories of both Windaus and Askew. Askew retained the term *calciferol* for the vitamin, while Windaus, realizing the possibility of the multiple nature of vitamin D designated it D_2.

The vitamin (calciferol) consists of clusters of colorless needles of melting-point 114.5 to 117. It is optically active, $[\alpha]_D^{20^\circ} + 102.5$ in alcohol. It is very soluble in most organic solvents. The antirachitic potency is 40,000 international units per milligram (p. 614).

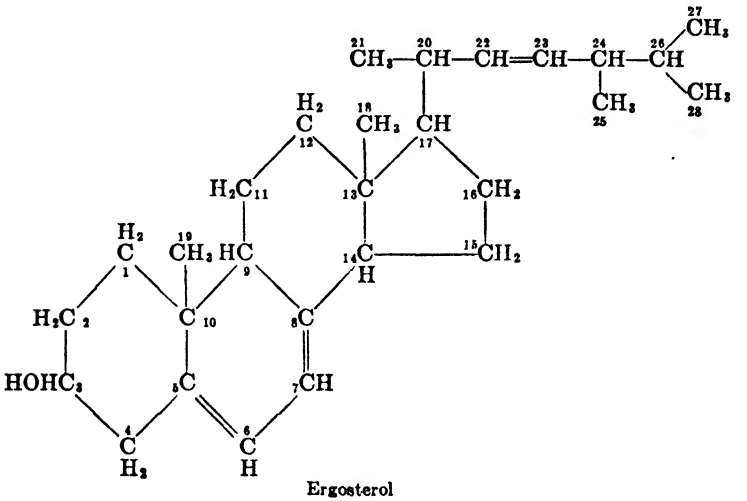
[264] C. E. Bills, E. M. Honeywell, and W. A. McNair, *J. Biol. Chem.*, **76**, 251 (1928); S. K. Kon, K. F. Daniels, and H. Steenbock, *J. Am. Chem. Soc.*, **50**, 2573 (1928); F. C. Koch, E. M. Koch, and I. K. Ragins, *J. Biol. Chem.*, **85**, 141 (1929); Koch, Koch, and H. B. Lemon, *ibid.*, **85**, 159 (1929).

[265] F. A. Askew and others, *Proc. Roy. Soc.* (*London*), *B*, **107**, 76, 91 (1930); **108**, 340 (1931); **109**, 488 (1931–32).

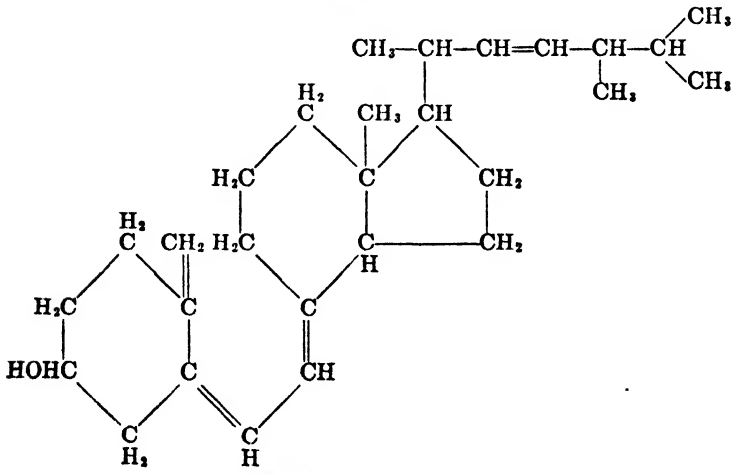
[266] A. Windaus and others, *Ann. Chem.*, **489**, 252 (1931); **492**, 226 (1932); **499**, 188 (1932); P. Setz., *Z. physiol. Chem.*, **215**, 183 (1933); F. Laquer and O. Linsert, *Klin. Wochschr.*, **12**, 753 (1933).

Irradiation of ergosterol results in the formation of a group of isomers. The first formed is believed to be *lumisterol*, which on further irradiation yields *tachysterol* and in turn vitamin D_2 (*calciferol*). Tachysterol does not possess antirachitic activity and is somewhat toxic. When given in excessive doses calciferol is likewise toxic. Vitamin D, on continued irradiation, yields *toxisterol* (*Substance* 248, so called because of its spectral absorption band at 248 mμ), and *suprasterols* I and II. As the name suggests, toxisterol is a toxic product.

As shown by the following structural formulas, calciferol is formed from ergosterol through the rupture of the C_9—C_{10} linkage.[267]



Ergosterol



Calciferol, or vitamin D_2

[267] A. Windaus and W. Thiele, *Ann.*, **521**, 160 (1935); A. Windaus and W. Grundman, *ibid.*, **524**, 295 (1936); Heilbron and associates, *J. Chem. Soc., London*, 905 (1935).

In order to clarify the situation at the outset, it should be stated that calciferol, or vitamin D_2, produced by irradiating ergosterol, though possessing marked antirachitic property, is not, as has been supposed until recently, the substance obtained by irradiating cholesterol, nor is it, in all probability, the form of vitamin D which is present in fish oils. Some of the evidence which has brought about this important revision in our conception will be summarized in the briefest possible form.

It will be recalled that Bills, Koch, and other investigators [264] could not rid cholesterol of at least some of its activatable material by bromination, a process designed to decompose ergosterol or similar unsaturated sterols. Eventually this fact, supported by other findings, contributed to the realization that the provitamin in cholesterol could not be ergosterol. About 1930, it was shown in Bills's laboratory [268] that irradiated ergosterol and the vitamin D of cod-liver oil behaved differently in rats and chicks. The two sources of antirachitic vitamin, standardized in terms of rat units and equal in potency as regards their effects in curing and preventing rickets in the rat, turned out to have widely divergent potencies in curing and preventing the comparable condition of leg weakness in chickens. It was found, in short, that rat unit for rat unit, the vitamin D of cod-liver oil was about 100 times as effective in chickens as the vitamin D of irradiated ergosterol. These observations were amply substantiated by other workers, and eventually this method of employing two species of animals (rat and chicken) for differential assay proved of inestimable importance in the further advance of the subject. In fact it was by utilizing this method that Waddell [269] was able to demonstrate in 1934 that irradiated cholesterol differed from irradiated ergosterol and that it was approximately as effective, rat unit for rat unit, as cod-liver oil for the chicken. The possibility that ergosterol irradiated in the presence of cholesterol might yield a vitamin having different properties from those of the one obtained by irradiating ergosterol alone was excluded by experimental evidence. It was therefore logical to conclude that the provitamin D of cholesterol was not ergosterol, as had been supposed, and that the vitamin obtained by irradiating cholesterol (from spinal cords) and the one derived from ergosterol (i.e., calciferol) were different substances.

Waddell's observations were confirmed in other laboratories, and further investigation made it seem probable that the activatable substance in cholesterol preparations was 7-dehydrocholesterol. In the meanwhile, the synthesis of this compound was accomplished by Windaus.[270] On irradiation it yielded a product which, like vitamin D of cod-liver oil and vitamin D of cholesterol, proved to be a more effective antirachitic agent for chicks than irradiated ergosterol. It was now also

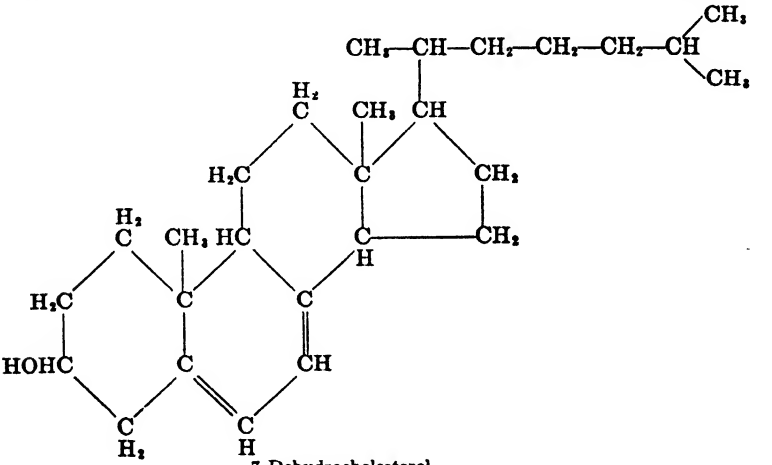
[268] O. N. Massengale and M. Nussmeier, *J. Biol. Chem.*, **87**, 423 (1930); see also O. N. Massengale and C. E. Bills, *J. Nutrition*, **12**, 429 (1936).

[269] *J. Biol. Chem.*, **105**, 711 (1934).

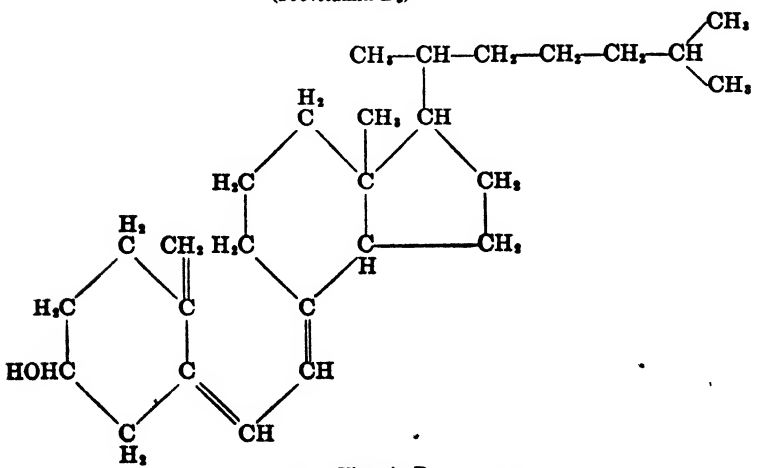
[270] A. Windaus, H. Lettré, and F. Schenck, *Ann.*, **520**, 98 (1935); Windaus, Schenck, and v. Werder, *Z. physiol. Chem.*, **241**, 100 (1936).

possible to explain why ergosterol had been mistakenly identified as the provitamin D in cholesterol preparations, for it was found that the spectral absorption bands of 7-dehydrocholesterol are very similar to those of ergosterol.

Irradiated 7-dehydrocholesterol was designated by Windaus as vitamin D_3. Was this antirachitic substance merely a laboratory product, or did it exist in nature? The question was definitely answered by Brockman [271] when he isolated the antirachitic principle present in tunafish-liver oil and found it to be identical with the irradiated 7-dehydrocholesterol obtained by Windaus and associates. Differential biological assay by the rat-chick method yielded confirmatory evidence. Below are given the structural formulas of provitamin and vitamin D_3.



7-Dehydrocholesterol
(Provitamin D_3)



Vitamin D_3
(Irradiated 7-dehydrocholesterol)

[271] *Z. physiol. Chem.*, **241**, 104 (1936).

Vitamin D_3, first known as a laboratory product, is the only naturally occurring antirachitic sterol that has been isolated so far, but it is practically a foregone conclusion that other natural forms of vitamin D exist, and it is probable that eventually at least some of these will be identified. The multiple nature of vitamin D has been brought out particularly by the work of Bills and associates,[272] who have made an exhaustive study of the liver oils of a large number of species of fish. Comparative bioassay has revealed that certain oils resemble cod-liver oil in their relative effects on the rat and chick, while other oils are either less effective, or more effective, in the chick, than cod-liver oil. The most convincing explanation of these results is based on the theory that any given fish oil probably contains at least two vitamins D, the proportions of which differ with the species.

Other Antirachitic Sterols and Their Provitamins. As far back as 1929, Koch and associates[264] made the important discovery that, if cholesterol (ordinary, as well as highly purified preparations) is heated to about 200° C. before irradiation, the resulting product is about twenty-five times more active than the one obtained by irradiation without preliminary heating. This result was eventually interpreted as signifying that, in the process of heating, a portion of the cholesterol is converted into an activatable substance. Later the work of Koch and other investigators[273] disclosed that irradiated, heat-treated cholesterol contains a vitamin which in its differential behavior toward rats and chicks resembles more closely the vitamin of fish-liver oils than does irradiated ergosterol. In a recent report, Koch and Koch[274] have submitted evidence that the provitamin D of *heated* purified cholesterol is *not* 7-dehydrocholesterol. They state, moreover, that spinal-cord cholesterol contains, besides 7-dehydrocholesterol, a second activatable contaminant which may be identical with the compound formed by heating cholesterol.

In 1933, Windaus[275] prepared 22-dihydroergosterol by introducing two atoms of hydrogen in the side chain of ergosterol (C_{22} and C_{23}). On irradiation it yielded an antirachitic substance (22-dihydrocalciferol), which proved to be more potent in the chick than vitamin D_2 (calciferol). McDonald[276] obtained an active product by irradiating 7-hydroxycholesterol, and McDonald and Bills[276] have reported the activation of a provitamin derived from sitosterol; this may be identical with the 7-dehydrositosterol isolated independently by Wunderlich.[277]

[272] C. E. Bills, O. N. Massengale, M. Imboden, and H. Hall, *J. Nutrition*, **13**, 435 (1937); C. E. Bills, *J. Am. Med. Assoc.*, **108**, 13 (1937).

[273] E. M. Koch and F. C. Koch, *Science*, **82**, 394 (1935); M. L. Hathaway and D. E. Lobb, *J. Biol. Chem.*, **113**, 105 (1936); R. W. Haman and H. Steenbock, *ibid.*, **114**, 505 (1936).

[274] E. M. Koch and F. C. Koch, *J. Biol. Chem.*, **116**, 757 (1936).

[275] A. Windaus and R. Langer, *Ann.*, **508**, 105 (1933).

[276] Cited by Bills, *J. Am. Med. Assoc.*, **108**, 13 (1937).

[277] *Z. physiol. Chem.*, **241**, 116 (1936).

Antirachitic sterol derivatives have also been prepared by chemical activation. Bills,[278] the pioneer in this field of research, found that cholesterol acquired antirachitic potency when treated with a clay catalyst, floridin, and that ergosterol could be activated when treated with nitrites. The subject has been actively pursued by Yoder and collaborators,[279] who have obtained active products from cholesterilene, dicholesteryl ether, cholesteryl chloride, cholesthene, and butyl cholesteryl ether on treatment with sulfuric acid and acetic anhydride. Irradiation did not convert these sterols into antirachitic compounds.

A number of the provitamins and their activated derivatives have been isolated and their chemical constitution determined; the identification of others is left to the future. Considering the rate and quality of recent progress, it is probable that further developments of fundamental importance will not be long delayed.

Summary. To recapitulate, irradiated 7-dehydrocholesterol (vitamin D_3) occurs naturally in fish-liver oils and has been isolated from tunafish-liver oil. It has also been prepared as a laboratory product. The existence of other forms of vitamin D in nature is strongly suspected.

Irradiated ergosterol, and more specifically calciferol, or vitamin D_2, is a potent antirachitic substance, but it has not been shown to occur in fish oils and other natural products. It is found, however, in the milk of cows that had been fed irradiated yeast and in the egg yolk of chickens that had been given irradiated ergosterol. In milk that has been irradiated directly, the increased antirachitic potency is probably due to the activation of 7-dehydrocholesterol.

Antirachitic compounds have been obtained by irradiation of the following sterols: 22-dihydroergosterol, 7-hydroxycholesterol, 7-dehydrositosterol. Besides these, there is the provitamin formed by heating cholesterol. The same provitamin may exist, along with 7-dehydrocholesterol, in ordinary cholesterol preparations. In addition to the products of irradiation, there is an increasing number of antirachitic compounds obtained by the action of chemical agents.

From the foregoing very brief presentation, it is seen that recent developments have brought into view a much broader horizon than was formerly perceived and have opened many new avenues for future research. In the attempt to determine the chemical nature of the antirachitic vitamin, it was discovered that physiologically active substances may be produced artificially from certain sterols, either by irradiation or by chemical means.

Nutritional Significance of Vitamin D. We do not have at the present time a fundamental understanding of the physiological action of vitamin D, although its relation to rickets has been definitely established. It is a relation which differs somewhat from the relation of

[278] *J. Biol. Chem.*, **67**, 753 (1926).

[279] *Ibid.*, **116**, 71 (1936); J. C. Eck, B. H. Thomas and L. Yoder, *ibid.*, **117**, 655 (1937).

vitamin C to scurvy, for example, or of vitamin B_1 to polyneuritis, for it seems that vitamin D deficiency does not inevitably lead to rickets and may be prevented, at least in a considerable measure, by providing adequate amounts of calcium and phosphate in the proper proportions. At the same time, it is to be said that vitamin D plays an important function in bone metabolism, and under ordinary circumstances its deficiency in children is associated with faulty bone formation. Depending upon the degree of its severity, the disease leads to various types of malformation, such as bow legs, deformed chest and skull, knock knees, etc. It is now generally conceded that any one or more than one of the following factors are significant: (*a*) calcium deficiency, (*b*) phosphorus deficiency, (*c*) improper balance between calcium and phosphorus, (*d*) lack or deficiency of vitamin D, (*e*) lack of direct sunlight which includes ultraviolet radiations. These factors are closely interrelated, and it even appears that the antirachitic vitamin may be completely replaced by ultraviolet light.

It has been demonstrated that vitamin D promotes the absorption, retention, and utilization of calcium and phosphorus. Compared to the normal, these elements are poorly absorbed in the rachitic animal or child, and owing to deficient retention and utilization, a greater proportion is excreted. These findings are reversed when sufficient vitamin D is given in rickets to induce healing. Along with improved absorption, there is increased retention and utilization and a lowered excretion of both elements.

In the adult, the manifestations of vitamin D deficiency may be slight, compared to the effects observed in the growing infant and child, or in the pregnant or nursing woman, but it is safe to assume that this factor is probably of importance in the nutrition of adults, especially of those kept on restricted diets.

Macy [280] and her associates have demonstrated the necessity of furnishing an adequate supply of vitamin D during pregnancy and lactation (in human cases) to prevent a negative calcium (or phosphate) balance. Inasmuch as milk is an inefficient source of the antirachitic vitamin, competent pediatricians now recognize the importance of administering this factor from earliest infancy. It has been determined that the vitamin content of cow's milk may be materially increased by feeding irradiated ergosterol, or better, irradiated yeast. The significance of vitamin D in tooth formation and the etiological relationship of vitamin D deficiency to such dental diseases as caries has received increasing attention in recent years.[281]

Reports have appeared showing that the administration of massive doses of irradiated ergosterol produces a condition of " hypervitaminosis " which is associated with diminished fecal excretion of calcium

[280] *J. Biol. Chem.*, **86**, 59 (1930); *ibid.*, **91**, 675 (1931).

[281] M. Mellanby and C. L. Pattison, *Brit. Med. J.*, **1**, 507 (1932); Mellanby, *ibid.*, **2**, 749 (1932); R. G. and M. C. Agnew, *J. Dental Research*, **11**, 478 (1931).

(and phosphorus), hypercalcemia, the widespread deposition of calcium in blood vessels and in various organs and tissues, other pathological changes, and death. Though it suggests the necessity for caution, this does not reflect against the therapeutic usefulness of irradiated ergosterol, for the quantities required to produce experimental hypervitaminosis are many thousand times as great as the amounts needed to produce the desired effect in experimental rickets.

Formation and Storage. It is probable that the synthesis of vitamin D occurs in fishes, although the evidence for this theory is at present inconclusive. The differences in the potency of various fish-liver oils may be ascribed to species differences in the ability to synthesize, rather than to differences in the ability to salvage and store vitamin D (Bills [282]). On the other hand, the higher animals depend largely on exogenous sources. According to Bills, there are at least three ways in which animals may obtain vitamin D; the relative importance of these vary with habits, requirements, and opportunities: "*a*, by eating such foods as eggs, fish, whole furred or feathered animals, and insolated dead vegetable tissues; *b*, by ingesting insolated sebaceous matter in the process of neatening the body—licking and preening; and *c*, by directly absorbing the products of insolation formed on or in the skin." Exclusive of the formation of antirachitic substance from its precursors in the skin by radiation, the higher animals are evidently incapable of synthesizing vitamin D, or at any rate in amounts sufficient to meet their needs. In birds, the sebaceous secretion is confined to the preen gland. It has been found in the case of chickens that, if this gland is extirpated, sunshine is no longer effective in preventing rickets.

The ability of the organism to store vitamin D has been repeatedly demonstrated, and it is also known that the vitamin reserve is depleted when the diet is changed to one that is devoid of this vitamin, the rate of depletion being greater if there is an accompanying deficiency of calcium and phosphorus. Heymann [283] has shown that following the administration of a single large dose of viosterol (200,000 U.S.P. units of vitamin D, corresponding to 1.6 mg. of irradiated ergosterol) to rabbits, some of the vitamin remained in certain tissues (liver, blood plasma) for as long as 12 weeks. In the brain it was stored for only 1 to 2 weeks; it remained in the skin for 6 to 8 weeks and in the kidneys for 6 to 9 weeks.

A transfer of vitamin D from the maternal organism to the fetus probably occurs, and it is definitely known that the vitamin D content of the mother's diet affects materially the antirachitic potency of the milk.

Assay and Standardization. The methods used in the estimation of the antirachitic potency of fish oils and other substances utilize the

[282] C. E. Bills, "Physiology of the Sterols, Including Vitamin D," *Physiol. Rev.*, **15**, 1 (1935).

[283] *J. Biol. Chem.*, **118**, 371 (1937).

rat and the chick as test animals and depend, for the most part, on calcification of bone as a criterion. The effect of a measured dose of a given preparation in inducing ossification in a rachitic rat may be determined with a fair degree of accuracy by X-ray examination of selected bones, as well as by means of the "line" test. In the latter procedure the bones are cut in two and the metaphyses examined usually after staining with silver nitrate. A more precise method applicable to both rats and chicks depends on the determination of the ash content of the bones.

The international standard consists of a solution of irradiated ergosterol, and the international unit is represented by 1 mg. of this solution. Its activity is such that, when one unit is administered daily for 8 successive days to a rachitic rat, a wide calcium "line" is formed. One international unit is equivalent to one U.S.P. unit of vitamin D.

The antirachitic potency of cod-liver oil averages 100 units per gram. The potency of pure crystalline calciferol (vitamin D_2) is 40,000 international units per milligram. Vitamin D_3 (irradiated 7-dehydrocholesterol) possesses about 24,000 units of vitamin D activity per milligram. These values are based on rat assay methods. Data concerning the vitamin D content of various fish oils have been published by Bills, Massengale, Imboden, and Hall.[284] The liver oils of the tunas, belonging to the order *Percomorphi*, were found to be especially rich in vitamin D (and also in vitamin A). The liver oil of California bluefin tuna was found to contain 46,000 international units (rat) of vitamin D per gram, while that obtained from the Japanese species of bluefin tuna contained 61,000 I.U. per gram.

Vitamin D and Its Requirements in Man. Quantitative data are lacking for most foods, and it is generally held that vitamin D has a rather limited distribution. Even such articles of food as milk and butter are described as relatively poor sources;[285] yet considerable amounts of vitamin D may be derived from dairy products and eggs if these are eaten in abundance. It is certain, however, that the diets of infants and children, as well as of women during pregnancy and lactation, should be supplemented with some rich source of vitamin D, such as cod-liver oil. The estimated requirements for infants and children is 1000 units in the form of cod-liver oil (or of certain other fish oils), or about 3000 units in the form of irradiated ergosterol. For pregnant and lac-

[284] *J. Nutrition*, **13**, 435 (1937).

[285] Several methods are employed commercially for increasing the vitamin D potency of milk. One procedure consists in fortifying the milk to a potency of about 400 I. U. per quart by the addition of irradiated ergosterol, or of a natural vitamin D concentrate. A second method consists in irradiating the milk itself, thereby increasing its vitamin content to about 135 I. U. per quart. A third procedure depends on feeding irradiated yeast to milch cows. The milk thus produced is often quite variable in respect to its antirachitic potency.

The vitamin D content of eggs may also be increased by feeding hens either irradiated yeast or a concentrate of vitamin D.

tating women, the requirement is probably 50 to 100 per cent higher. More definite knowledge than is now available is needed concerning the vitamin D requirement in adult nutrition.

Vitamin E; the Reproductive Factor in Nutrition

The existence of a specific reproductive factor was established through the independent observations of Evans,[286] Mattill,[287] Sure,[288] and their associates. Rats maintained on diets adequate in other respects, but deficient in the fat-soluble vitamin E, developed sterility, the effect of the deficiency differing in the two sexes. In the male as shown by Mason,[289] as well as by Beard [290] and others, vitamin E deprivation leads to degenerative changes of the germinal epithelium. A similar effect is produced by vitamin A deficiency, but the difference lies in the fact that the testicular changes due to avitaminosis A are reparable, fertility being restored on subsequent vitamin A administration, whereas the damage produced (in the male) by vitamin E deprivation is irrevocable.

In the female, vitamin E deficiency affects neither the ovaries nor the ova; indeed, there is not even interference with the earlier stages of gestation. The embryos develop normally for about five days, or even longer, after which they succumb, fetal resorption then taking place. A single dose of vitamin E, administered as late as the fifth day, permits the continuation of the pregnancy and its ultimate successful conclusion. It thus appears that vitamin E deficiency primarily affects the embryo and not the maternal organism.

As regards the reproductive process, it is evident that not only vitamin E, but each of the other known vitamins, exerts its own special influence.

Occurrence of Vitamin E. The absence of the antisterility factor at least in certain brands of cod-liver oil led to its early discovery, for it was observed that rats supplied with cod-liver oil as the source of vitamins A and D characteristically developed sterility. Vitamin E is found in the unsaponifiable, non-sterol fraction of oils derived from grains, vegetables, and other sources. As we shall see, it is multiple in nature. Wheat germ oil is the richest known source. Cottonseed, corn, palm, and other oils contain an abundance of the vitamin; certain others (linseed, coconut, palm kernel, commercial corn oil) are relatively deficient in this respect. Egg yolk is a good source; milk is a relatively poor one. The green leaves of watercress, lettuce, spinach, and alfalfa are regarded as very good sources. Muscle and body fats contain more

[286] H. M. Evans and K. S. Bishop, *J. Metabolic Research*, **1**, 319 (1922). For a recent review of the subject, the reader is referred to Evans, *J. Am. Med. Assoc.*, **99**, 469 (1932).

[287] H. Mattill and N. C. Stone, *J. Biol. Chem.*, **55**, 443 (1923).

[288] *Ibid.*, **58**, 693 (1924).

[289] *J. Exptl. Zool.*, **45**, 159 (1926).

[290] *Am. J. Physiol.*, **75**, 682 (1926).

than liver, kidneys, or gonads, the last being surprisingly low in this factor.

Chemical Properties of Vitamin E. Early in the study of the chemistry of vitamin E it was noted that the oxidative changes in fats associated with the development of rancidity also destroy the vitamin. It was also observed that wheat germ oil possesses a marked protective action against such oxidation. This association of vitamin E and the anti-oxidant effect of wheat germ oil and other oils (lettuce, tomato, carrot, alfalfa, cottonseed, corn, sesame, palm, soybean, peanut) has been the subject of intensive and fruitful investigation in Mattill's laboratory,[291] where it was suspected (Olcott and Mattill) that both properties were due to the presence of compounds having an alcohol (hydroxy) group. For this reason they designated the compounds associated with the inhibition of oxidation as *inhibitols*. No demonstrable amounts of inhibitols were found in yeast, lard, or in cod-liver, palm kernel, and castor oils.

Despite the similarity in distribution of vitamin E and inhibitols, Olcott was at first of the opinion that the two properties were due to distinct substances. Some vitamin E concentrates which he prepared also possessed marked anti-oxidant effects; others were virtually without this property.

It is of interest that the inhibitol concentrates prepared by Olcott and Mattill exhibited a strong absorption band with a maximum at 2940 Å, the intensity of which was proportional to the anti-oxidant activity of the preparations. The same spectral absorption had been previously noted by Drummond and associates,[292] working with vitamin E concentrates. These investigators determined that there was a parallelism between the intensity of absorption and the vitamin E activity.

Pursuing the problem further, Evans, Emerson, and Emerson [293] treated a vitamin E concentrate, obtained from wheat germ oil, with cyanic acid, a reagent which combines with alcohols, and obtained three allophanates. On hydrolysis an oily alcohol was obtained from which were eventually isolated three isomeric compounds of the composition $C_{29}H_{50}O_2$. In their first publication Evans and co-workers associated marked physiological activity with only one of these compounds, which was named α-tocopherol (*tokos* = childbirth; *pher* = bear; *−ol*, indicating alcohol). It was later shown that all three isomers, α-, β-, and γ-tocopherols, derived from wheat germ oil and subsequently from

[291] H. A. Mattill and B. Crawford, *Ind. Eng. Chem.*, **22**, 341 (1930); H. S. Olcott and H. A. Mattill, *J. Biol. Chem.*, **93**, 59, 65 (1931); **104**, 423 (1934); Olcott, *ibid.*, **107**, 471 (1934); **110**, 695 (1935); *J. Am. Chem. Soc.*, **58**, 1627, 2204 (1936).

[292] J. C. Drummond, E. Singer, and R. J. McWalter, *Biochem. J.*, **29**, 456 (1935); see also Drummond and A. A. Hoover, *ibid.*, **31**, 1852 (1937).

[293] *J. Biol. Chem.*, **113**, 319 (1936); *Science*, **83**, 421 (1936); Emerson, Emerson, Mohammed, and Evans, *J. Biol. Chem.*, **122**, 99 (1937).

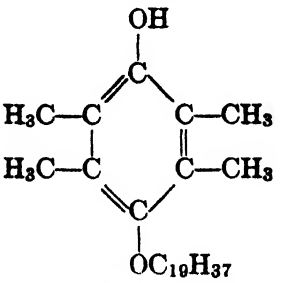
cottonseed oil, are physiologically active. α-Tocopherol shows a characteristic absorption band at 2980 Å.

Olcott and Emerson [294] then compared the vitamin E activity and anti-oxidant (inhibitol) properties of the three tocopherols and found that the α and γ forms possess approximately the same vitamin potency. In the case of each compound, 1–3 mg. was the approximate effective dose when administered to vitamin E deficient female rats. β-Tocopherol proved to be somewhat less active, the required dose being 3–5 mg. On the other hand, the activity of the tocopherols as anti-oxidants was found to increase in the order: α, β, γ.

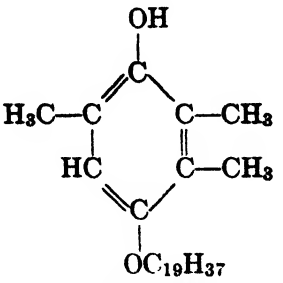
Because of the anti-oxidant property and the similarity of their absorption spectra to that of hydroquinone, Olcott and Emerson surmised that the tocopherols were perhaps related to this compound. This supposition was borne out almost immediately by the work of Fernholz,[295] who isolated durohydroquinone ($C_{10}H_{14}O_2$) from among the decomposition products obtained by heating α-tocopherol.

Recently, John [296] reported the isolation from wheat germ oil of two isomeric forms of a compound called cumotocopherol, $C_{28}H_{48}O_2$, a homologue of tocopherol. On thermal decomposition it yielded pseudocumolhydroquinone, $C_9H_{12}O_2$. The biological activity of cumotocopherol is less than that of tocopherol, approximately 8 mg. being the effective antisterility dose in the female rat. From this it is seen that the vitamin E (and anti-oxidant) effects are in all probability attributable to a group of chemically related compounds. Future work may disclose the existence of additional compounds of this type and, possibly, differences in their distribution in nature.

The chemical configuration of the side chain has not been definitely established, as yet. The constitution of α-tocopherol and of one of the isomeric forms of cumotocopherol may be represented tentatively by the following formulas:



α-Tocopherol



Cumotocopherol

Nutritional Significance of Vitamin E. The relation of vitamin E to sterility in the rat is definitely established. Inasmuch as this vitamin

[294] *J. Am. Chem. Soc.*, **59**, 1008 (1937).
[295] *Ibid.*, **59**, 1154 (1937).
[296] *Z. physiol. Chem.*, **250**, 11 (1937).

is so widely distributed in nature, it is not considered probable that its deficiency in man and animals would be encountered ordinarily. However, Vogt-Møller [297] reported having obtained a curative effect in spontaneously occurring sterility in cattle through the administration of vitamin E. He also reported favorable results in two women, who, after repeated miscarriages, each gave birth to a live child. These reports notwithstanding, the status of vitamin E in human and animal sterility is at present unsettled.

Antihemorrhagic Factor, or Vitamin K

The work of a number of investigators [298] has led them to postulate the existence of a fat-soluble factor, specifically involved in blood coagulation in chicks, ducklings, goslings, and possibly other birds. Deficiency of this nutritional factor is said to result in a prolonged blood-clotting time, a marked tendency to hemorrhage (subcutaneous, intramuscular, abdominal, etc.), and anemia. The antihemorrhagic factor occurs in vegetables, especially of the leafy variety, in hog-liver fat, and to a lesser degree in the oils of grains and cereals. It is a constituent of the non-sterol fraction of the unsaponifiable matter.

Almquist has recently reported the isolation of the antihemorrhagic substance in crystalline form, using as the starting material a concentrate prepared from dried alfalfa.

It has been suggested that vitamin K may be the prosthetic group of prothrombin, but otherwise practically nothing is known concerning its physiological action. Judging from the reports of Almquist and others, vitamin K is essential in poultry nutrition. It is to be recognized that the discovery of this principle is of such recent occurrence that more time must be allowed and additional information gathered before it will be possible to evaluate more fully its nutritional importance.

SUMMARY

In this chapter the requirements of proper nutrition have been considered. It has been pointed out that the organism should receive an adequate amount of food to supply its calorific needs. A sufficient supply of inorganic elements is likewise imperative. Fortunately, our ordinary food contains many of these in amounts that are in excess of the natural requirements, but frequently the supply of such elements as

[297] *Lancet*, **2**, 182 (1931).

[298] H. Dam, *Nature*, **135**, 652 (1935); *Biochem. J.*, **29**, 1273 (1935); F. Schønheyder, *Nature*, **135**, 552 (1935); Dam and Schønheyder, *Biochem. J.*, **30**, 897 (1936); Dam and L. Lewis, *ibid.*, **31**, 17, 22 (1937); H. J. Almquist and E. L. R. Stockstadt, *J. Biol. Chem.*, **111**, 105 (1935); *J. Nutrition*, **12**, 325 (1936); H. J. Almquist, *J. Biol. Chem.*, **114**, 241; **115**, 589 (1936); **120**, 635 (1937); *Poultry Sci.*, **16**, 166 (1937). Clinical studies with vitamin K have been recently reported: *Proc. Staff Meetings Mayo Clinic*, **13**, 65 (1938).

calcium, phosphorus, iron, and iodine may be deficient. The protein of the diet should be adequate, both from the standpoint of quantity and from that of quality. The biological value of proteins depends upon the presence of certain essential amino acids, without which the organism is unable to restore its worn-out tissue or to maintain nitrogen equilibrium. As we have seen, knowledge of the rôle of the amino acids in nutrition has been advanced enormously through the use in experimental studies of mixtures of pure amino acids and the discovery of all the indispensable dietary components of the protein molecule.

The growth of knowledge of the vitamins represents one of the most significant developments of modern science. Less than thirty years ago their existence was barely suspected, and until recently it was customary to define them as compounds of unknown chemical constitution. Today not only do we possess information concerning their specific functions in nutrition, but practically all of the known vitamins have been isolated and their chemical composition determined, and many of them have either been synthesized, or prepared in the laboratory from related chemical compounds. It has been discovered that a given vitamin (vitamin A, for example) may have more than one natural precursor, or provitamin, and that properties once attributed to a single substance (vitamin D or E) are possessed, in varying degree, to be sure, by several chemically related compounds. From the original limited conception of vitamin B has evolved the more complete understanding of the different components of the "vitamin B complex." The demonstration that at least certain of the vitamins, recognized originally by their effects in preventing certain diseases, are also components of enzyme systems has broadened immensely our perspective of the subject and has brought it into closer relation to fundamental considerations.

A superficial view of the situation may produce the impression that the vitamin problem has become very confusing. But, on closer study, it will be realized that, despite the rapidity with which new ideas have been projected and the intricate relations that have been revealed, the problem as a whole has been definitely clarified. We have, certainly, a more complete and tangible conception of the vitamins than formerly, not only as regards their chemical properties and nutritional importance, but also as regards at least a few of the underlying physiological properties. It is to be expected that future research will broaden our knowledge and lead to a more complete integration of the various aspects of the subject.

The problem of nutrition has other features which have not been considered and which in fact have been observed so recently that their significance is not yet fully appreciated. Thus, a new outlook has been created by the studies of Sherman and Campbell,[299] which indicate that a diet that is more than adequate, in the accepted sense, may

[299] *Proc. Natl. Acad. Sci., U. S.*, **14**, 852 (1928); *J. Nutrition*, **2**, 415 (1930).

have a direct influence in prolonging the normal life-span of experimental animals.[300] In addition to this possible relationship of diet to longevity which would concern the welfare of the individual, there is perhaps another relationship of even greater biological significance. It has been the experience of those[301] who have been engaged for many years in the study of nutrition and who have had an opportunity to observe colonies of white rats through many generations that under favorable dietary and environmental conditions the individuals of successive generations grow more rapidly, are larger and more flourishing. While the elements of selection and heredity are not to be excluded, there is obviously a nutritional factor which is responsible for the improvement of the stock. Those who are interested in individual and racial physical betterment may well be guided by scientific observations such as these.

[300] It should be noted that any dictum, however plausible it may seem, is to be regarded with extreme caution, unless supported by an abundance of incontrovertible evidence. To illustrate, there is the possibility that the best interests of the individual may not be served by an abundant, or superabundant, diet early in life. McCay and Crowell have observed that rats whose growth was purposefully retarded by inadequate calories and which therefore attained maturity later than normal, were, however, physiologically younger and had a much longer life-span than the unretarded, rapidly, or "normally" growing rats. From their study they were also led to conclude that the potential life-span of an animal species is unknown and greater than has been believed (*Scientific Monthly*, **39**, 415 [1934]).

C. M. Jackson's observations are also of interest in this connection. Rats, after weaning, were kept at nearly constant weight (about 50 grams) for 15 weeks by a protein-deficient diet, then refed on normal stock diet. Compared to litter-mate controls, kept on stock diet throughout, the test animals, especially the males, showed a high mortality, but, with the change in diet, the survivors gained weight more rapidly than normal, so that at 9 months of age the female test animals excelled their female controls. The males lagged behind their controls. However, certain anatomical abnormalities (weights of organs, etc.) were not corrected by the change in dietary regimen. *Am. J. Anat.*, **58**, 179 (1936).

[301] T. B. Osborne and L. B. Mendel, *J. Biol. Chem.*, **69**, 661 (1926); Mendel and Cannon, *ibid.*, **75**, 779 (1927); see also A. H. Smith and F. C. Bing, *J. Nutrition*, **1**, 179 (1928); W. E. Anderson and A. H. Smith, *Am. J. Physiol.*, **100**, 511 (1932).

CHAPTER XIX

THE COMPOSITION OF MILK AND CERTAIN TISSUES

Milk. The young mammal depends for its nourishment almost exclusively upon milk, which despite certain deficiencies is nevertheless probably the most complete single food found in nature. It contains protein, fat, the sugar lactose, inorganic salts, organic acids, certain non-protein nitrogenous constituents, and vitamins, of which A and G (B_2) are present in abundance, and B_1 C, and D, in smaller amounts, as has been stated in other connections.

Milk is normally slightly acid in reaction, having a *p*H of approximately 6.6 to 6.9.

Of the proteins in cows' milk, all but about 15 per cent is casein. The remainder is lactalbumin together with a small amount of lactoglobulin and traces of other proteins. In human milk, in which the protein content is considerably less than in cows' milk, being ordinarily about 1 per cent, there is, according to Macy's data,[1] approximately equal distribution between casein and the remaining proteins. The protein of milk is derived from the amino acids of the blood, the synthesis occurring in the mammary glands.[2]

About 90 per cent of milk fat is composed of the glycerides of the higher fatty acids, including myristic, palmitic, stearic, and oleic. The remainder consists of the glycerides of the lower fatty acids, butyric, caproic, caprylic, capric, and lauric. Milk fat is unique in containing the complete range of fatty acids from C_4 to C_{26}. The constituent fatty acids of human milk have been investigated by Bosworth.[3] Very small amounts of other lipids are present in milk, including lecithin, cephalin, cholesterol, and free fatty acids. The theory formerly held that milk fat is derived from the phospholipids of the blood has been abandoned by a number of contemporary writers.[4]

[1] I. G. Macy and others, *Am. J. Diseases of Children*, **39**, 1186 (1930); **42**, 569 (1931); **43**, 40, 828, 1062 (1932). For a review of the nutritive aspects of milk, the reader is referred to Macy, *Yale J. Biol. Med.*, **4**, 451 (1932); see also B. N. Erickson, *et al.*, *J. Biol. Chem.*, **106**, 145 (1934).

[2] C. A. Cary, *J. Biol. Chem.*, **43**, 477 (1920); J. H. Blackwood and J. D. Stirling, *Biochem. J.*, **26**, 772, 778, 1127 (1932); W. R. Graham, *et al.*, *J. Biol. Chem.* **122**, 275 (1938).

[3] *J. Biol. Chem.*, **106**, 235 (1934).

[4] J. H. Blackwood, *Biochem. J.*, **28**, 1346 (1934); R. G. Sinclair, *Ann. Rev. Biochem.*, **6**, 263 (1937).

The lactose of the milk (milk sugar) is derived from the glucose of the blood.[5]

The inorganic salts and other constituents are likewise derived from the blood, some by a process of simple filtration. The ash content of milk varies in different mammals, being, for example, much higher in cow's than in human milk. The elements contained in the ash are: Ca, P, K, Na, Mg, S, Cl, and traces of Fe, I, Cu, Zn, etc.[6] The amounts in which the more important of these are present are indicated by the following data:[7]

TABLE LX

	Cow's Milk, Per Cent	Human Milk, Per Cent
Phosphorus (inorganic).....	0.087	0 0148
Calcium.....	0.144	0 0354
Magnesium.....	0 013	0 0030
Potassium.....	0 120	0 0711
Sodium.....	0.055	0 0147
Chlorine.....	0.076	0 0711
Total ash, average.....	0.725	0 30±

The factors which influence the yield and composition of cow's milk are: breed, age, stage of lactation, frequency of milkings, diet, pain, anxiety, fatigue, etc.[8] The data in Table LXI show the limits of variation, as well as the average values, for the composition of cow's and goat's milk.[9]

Human milk is likewise influenced by various factors. These have been studied exhaustively in a limited number of individuals by Macy and associates (Hunscher, Donelson, Nims, etc.).[1] They observed marked individual variation; one subject secreted over 2000 cc. daily, and not infrequently more than 3000 cc., whereas another subject

[5] M. Kaufman and H. Magne, *Comp. rend.*, **143**, 779 (1906); J. H. Blackwood and J. D. Stirling, *Biochem. J.*, **26**, 362 (1932); G. A. Grant, *Biochem. J.*, **29**, 1905 (1935); **30**, 2027 (1936).

[6] In addition to these elements Wright and Papish (*Science*, **69**, 78 [1929]) have reported the detection, spectroscopically, of traces of the following elements: Al, Mn, Si, B, Ti, Vd, Rb, Li, and Sr.

[7] According to Bosworth (*J. Biol. Chem.*, **20**, 707 [1915]), the probable condition of these constituents in human milk is as follows: Calcium, in combination with protein, 0.014 per cent; calcium chloride, 0.059 per cent; monopotassium phosphate, 0.069 per cent; sodium citrate, 0.055 per cent; potassium citrate, 0.0103 per cent; monomagnesium phosphate, 0.027 per cent.

[8] Consult, E. B. Meigs, "Milk Secretion as Related to Diet," *Physiol. Rev.* **2**, 204 (1922).

[9] A. E. Leach, "Food Inspection and Analysis," revised by A. L. Winton, 4th ed., John Wiley & Sons, 1920, p. 113; see also Winton, "Structure and Composition of Foods," Vol. III, John Wiley & Sons, 1937.

rarely exceeded 1500 cc. The composition was approximately the same in both cases. Significant variations in the volume output of individual women were also observed from day to day and from hour to hour. As emphasized by Macy, such fluctuations may have an appreciable influence on the nurturing of the average breast-fed infant. It was found that frequent and complete emptying of the breasts encouraged the flow of milk, as did a liberal protein intake, provided, of course, that the food was otherwise adequate and well balanced. The flow of milk was reduced by excessive exercise and heavy work, and even moderate exercise had a depressing effect. Milk flow is also suppressed by emotional disturbance, such as excitement, fear, anxiety.

TABLE LXI

	No. of Analyses	Specific Gravity	Water	Fat	Lactose	Total Protein	Casein	Albumin	Ash	Fuel Value per Lb., Calories
Cows' milk....	800									
Maximum...		1 0370	90.32	6.47	6.12	6 40	6 29	1 44	1.21	
Minimum...		1 0264	80 32	1.67	2.11	2.07	1.79	0 25	0.35	
Average....		1 0315	87.27	3.64	4.88	3 55	3 02	0.53	0.71	310
Goats' milk....	200									
Maximum...		1 0360	90 16	7 55	5.77	....	3 94	2.01	1.06	
Minimum...		1.0298	74.47	2 81	2.76	....	3.59	0 83	0 13	
Average ...		1 0305	85 71	4.78	4 46	4 29	3 20	1 09	0.76	364

During the progress of a single nursing, the fat, protein, and total solids tend to increase. Macy found that the two breasts functioned differently in the total production of milk and milk nutrients, but the concentrations of the various constituents were the same in the two secretions. Fluctuations in composition were noted from day to day, but a more definite trend occurred as the period of lactation progressed, especially as regards the decrease of the protein and ash content.

Human milk differs from cows' milk in having less casein and ash, more lactose, and a greater proportion of albumin.[10]

The secretion produced by the mammary glands for two to four days after the birth of the young is termed *colostrum*. It is a yellowish, alkaline fluid of greater viscosity and specific gravity than milk and has a much higher content of total solids, which in cows' colostrum may exceed 25 per cent. Albumin is the chief constituent, frequently form-

[10] Leach[9] gives the following data for 94 analyses of human milk: Specific gravity, max. 1.0426, min. 1.024, av. 1.0313; water, av. 88.2 per cent; fat 9.05–0.47, av. 3.3 per cent; lactose, 8.89–4.42, av. 6.8 per cent; total protein, 5.56–0.85, av. 1.50 per cent; ash, 0.5–0.09, av. 0.20 per cent; average fuel value in calories per pound, 295.

ing more than 15 per cent of the colostrum. Colostrum exerts a purging effect on the new-born mammal.

Human colostrum contains 8 to 10 per cent protein and more inorganic constituents and less lactose and fat than milk. From the fifth day *post partum* until the end of the first month the milk shows a gradual change in composition, the protein and ash contents diminishing, whereas the amounts of lactose and fat tend to increase (Bell[11]). The limits of variation as well as the average composition of human milk at different periods are shown by the data in the following table (after Bell):

TABLE LXII

AVERAGE COMPOSITION OF HUMAN MILK AT DIFFERENT PERIODS

Time	No. of Cases	Protein			Sugar			Fat		
		Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average
		Per Cent	Per Cent	Per Cent	Per Cent	Per Cent	Per Cent	Per Cent	Per Cent	Per Cent
5 days	88	1.45	2.83	2.00	4 62	7 37	6 42	0 9	8.2	3.2
9 "	88	1 12	2.65	1 73	4 76	7 65	6 73	1 6	7.1	3.7
3–4 wks.	35	1.03	1.79	1.37	6 17	7 89	7 11	1.4	6.1	3.6
5–6 "	32	0.98	1 57	1 30	5 97	8 33	7 11	1 3	7.6	4.0
7–8 "	14	1.04	1.40	1.21	6 25	7 83	7 11	1 1	7.0	4 0

Connective Tissue and Cartilage. Connective tissue contains approximately 60 per cent water and 40 per cent solids. Of the latter, about 0.5 per cent consists of inorganic matter. The principal organic constituent of white fibrous connective tissue is the albuminoid collagen, which composes about 32 per cent of the tissue, the remaining 6–7 per cent being made up of elastin, mucoid, ether-soluble lipids, coagulable protein, and non-protein nitrogenous constituents, or extractives.

The composition of connective tissue varies somewhat with age, the tissue of younger animals containing more water and mucoid and less collagen than that of older animals. On hydrolysis, collagen is changed to gelatin.

The principal constituent of yellow elastic tissue is the albuminoid elastin, which forms about 30–32 per cent of the tissue. About 7 per cent of collagen is also present. The remaining constituents are the same as those found in white fibrous tissue.

Collagen is likewise a constituent of cartilage, which contains in addition chondromucoid, chondroitin-sulfuric acid, and another albuminoid.

[11] M. Bell, *J. Biol. Chem.*, **80**, 239 (1928); see also I. S. Kleiner, J. E. Tritsch, and L. G. Graves, *Am. J. Obstet. Gynecol.*, **15**, 172 (1928).

The following data are typical of the composition of cartilage:

	Per Cent
Water	68–74
Solids	26–32
Organic matter	25–30
Inorganic matter [12]	1.5–2

Considerable variation in composition may be shown by cartilage from different parts of the body. Logan [12] has shown that the conversion of cartilage to the organic matrix of bone is characterized by loss of organic sulfates and gain of nitrogenous constituents.

Bone. Bone which is free from marrow contains 20 to 25 per cent of water. The organic matrix resembles the matrix of cartilage.[13] It consists principally of *ossein* which seems to be identical with collagen, a mucoid, *osseomucoid,* and an albuminoid. These comprise about 40 per cent of normal, dried, marrowless bone, the remaining 60 per cent consisting almost entirely of calcium in combination with phosphate and carbonate.[14]

It was shown by Taylor and Sheard,[15] that the solid inorganic phase of bone consists essentially of small crystals of mineral of the *apatite* group and may therefore be designated by the general formula $3Ca_3(PO_4)_2 \cdot CaX_2$, where X_2 ordinarily represents CO_3, but may also represent F_2, $(OH)_2$, O, SO_4, etc. Taylor and Sheard's conclusions were based on the chemical analysis of the inorganic phase of bone and of apatite and the resemblance of their X-ray diffraction patterns and refractive indices.

Hastings and associates [16] have also concluded that the inorganic phase of bone is essentially tertiary calcium phosphate containing calcium carbonate in solid solution, approximately in the proportion of 2 moles of $Ca_3(PO_4)_2$ to 1 mole of $CaCO_3$. Bone salt, therefore, is prin-

[12] M. A. Logan obtained the following values for the inorganic composition of cartilage (values in cubic centimeters of 0.1 *N* per 100 grams): Ca 17.8–25.2, Mg 7.5–14, Na + K 200–402, inorganic P 3.2–7.5, CO_2 10.4–14.4, Cl 46.0–53.8 (*J. Biol. Chem.*, **110**, 375 [1935]).

[13] For a brief though comprehensive account consult A. W. Ham, "Cartilage and Bone," in Cowdry's "Special Cytology," 2d edition, 1932, Vol. II

[14] The composition of the inorganic phase of bone is remarkably constant for different individuals of a given species, and, as originally shown by Gabriel, is almost the same for different animals. The following values in per cent have been obtained for human bone: CaO, 51.31; MgO, 0.77; K_2O, 0.32; Na_2O, 1.04; water of crystallization, 2.46; P_2O_5, 36.65; CO_2, 5.86; Cl, 0.01; water of constitution, 1.32. In addition very small amounts of fluoride are commonly found both in teeth and bone. *Z. physiol. Chem.*, **18**, 257 (1894); cited by R. Robison, "The Significance of Phosphoric Esters in Metabolism," New York, 1932, p. 42; see also M. Logan; [13] C. M. Burns and N. Henderson, *Biochem. J.*, **29**, 2385 (1935); **30**, 1202 (1936), and C. Huggins.[17]

[15] *J. Biol. Chem.*, **81**, 479 (1929).

[16] H. H. Roseberry, A. B. Hastings, and J. K. Morse, *ibid.*, **90**, 395 (1931); L. J. Bogert and A. B. Hastings, *ibid.*, **94**, 473 (1931–32); *New England J. Med.*, **216**, 377 (1937).

cipally carbonate-apatite, or *dahlite*. Other theories concerning the composition of bone salt have been reviewed recently by Huggins.[17]

According to Logan and Taylor [18] the formation of bone salts occurs in more than one step, the first being precipitation of tricalcium phosphate. This in turn removes from the liquid phase ions of other salts, such as calcium carbonate. Accordingly, newly formed bone has a lower proportion of carbonate and a higher proportion of phosphate than mature bone (Logan,[12] Burns and Henderson [14]).

Logan and Taylor have shown that precipitation of tricalcium phosphate from solution occurs spontaneously when the ion product, $[Ca^{++}]^3 \times [PO_4^{=}]^2$ exceeds the value $10^{-23.5}$. Once precipitation has been initiated, it will continue until the ion product is diminished to 10^{-27} provided that the solution is in contact with a sufficient amount ($>$ 150 mg. per liter) of solid phase composed of bone salt, $Ca_3(PO_4)_2$, or bone. Reduction of the ion product below 10^{-27} is prevented because at such concentrations bone salt will go into solution. On the other hand, if the ion product of the solution in immediate contact with bone salt is increased above 10^{-27}, more $Ca_3(PO_4)_2$ will precipitate. It may be assumed, therefore, that the ion product, $[Ca^{++}]^3 \times [PO^{=}_4]^2$, of fluid (blood plasma, for example) in immediate contact with bone is 10^{-27}.

For blood serum and other body fluids the ion product is normally in the neighborhood of $10^{-23.5}$. As shown by Logan and Taylor, spontaneous precipitation does not occur at this concentration if the fluid is not in contact with a solid phase composed of bone salt. This means that ordinarily the body fluids are *not* supersaturated in respect to calcium and phosphate ions. It is necessary to emphasize this point, as hitherto the opposite view has been generally held, the basis for it being that the concentrations of these ions could be lowered by equilibrating plasma (and other body fluids) with comparatively large amounts of solid tertiary calcium phosphate.

The composition of the inorganic phase is essentially the same in pathological calcifications as it is in normal bone, as has been shown by Wells [19] and others.[20]

Several theories have been advanced in the endeavor to explain the mechanism of calcification in bone. These have been recently summarized by Robison,[21] who advanced the conception that the

[17] *Physiol. Rev.*, **17**, 119 (1937).

[18] *J. Biol. Chem.*, **119**, 293 (1937); *Proc. Soc. Biol. Chem., ibid.*, **119**, xliv (1937).

[19] *Arch. Internal Med.*, **7**, 721 (1911); "Calcification and Ossification," *Harvey Lectures*, 1910–11, p. 102; "Chemical Pathology," Saunders, Philadelphia, 1925, p. 487.

[20] The subject of pathological calcification has been more recently reviewed by D. P. Barr, *Physiol. Rev.*, **12**, 593 (1932). See also B. Kramer and M. J. Shear, *J. Biol. Chem.*, **79**, 147 (1928); **83**, 697 (1929).

[21] Robison, R., "The Significance of Phosphoric Esters in Metabolism," New York Univ. Press, 1932; see also J. S. F. Niven and R. Robison, *Biochem. J.*, **28**, 2237 (1934); H. B. Fell and Robison, *ibid.*, **28**, 2243 (1934).

enzyme *phosphatase*, which occurs particularly in bone, teeth, kidney, and intestine, and is capable of hydrolyzing organic phosphoric acid esters, plays a significant rôle in the process. Robison observed that when bone from a rachitic rat was immersed in a solution of calcium hexosemonophosphate, or calcium glycerophosphate, there eventually resulted (at 37°) a deposition of calcium phosphate in the zone of provisional calcification. He attributed this to the action of phosphatase in liberating $PO_4^{=}$ from the ester, thereby increasing the ion product of $[Ca^{++}] \times [PO_4^{=}]$ at the site of calcification.[22]

Phosphatase is a normal constituent of plasma (or serum). Its content or activity is greatly increased in certain diseases of bone, such as clinical hyperparathyroidism (Recklinghausen's disease), Paget's disease, osteomalacia, and notably in active rickets.[23] Although the full significance of these changes is not known at present, they obviously reflect, even more than changes in the concentration of serum calcium and phosphorus, a disturbance in bone metabolism.

Teeth. In chemical composition the *cement* and *dentine* of teeth resemble bone, though the dentine contains less water. The *enamel*, which is a derivative of epithelium, contains still less water, only about 5 per cent, and is the hardest structure in the body. It differs from bone in having a higher phosphorus content and a somewhat different organic matrix, for on boiling with water, enamel does not yield gelatin as does bone. The protein of enamel shows the characteristics of keratin.

The composition of the enamel and dentine of sound human teeth, of good structure, as recently reported by Bowes and Murray,[24] is outlined in Table LXIII (p. 628).

From these values Bowes and Murray have computed that about 80 per cent of the calcium in enamel is combined in the form of hydroxyapatite and 12 per cent as carbonatoapatite (dahlite). Dentine contains

[22] Phosphate is absent from the unincubated egg. Its appearance in the embryonic femora and limb buds is coincident with the beginning of ossification. A similar correlation between the advent of phosphatase and the beginning of ossification has been described in the case of the human patella (Martland and Robison, *Biochem. J.*, **18**, 1354 [1924]). Huggins (*Arch. Surgery*, **22**, 577 [1931]; *Biochem. J.*, **25**, 728 [1931]) has shown that, when bladder epithelium is transplanted to the rectus sheath, the connective tissue adjacent to the transplant frequently ossifies, forming calcified bone. He has observed that the development of this heterotopic bone coincides with the production of phosphatase, in very high degree. The fact that the blood contains a suitable substrate for bone phosphatase in the form of the acid-soluble phosphoric esters is another point in favor of the view that this enzyme participates in bone metabolism.

On the other hand, it has been shown that the cartilagenous skeleton of the elasmobranch fish contains considerable amounts of phosphatase (O. Bodansky, R. M. and H. Bakwin, *J. Biol. Chem.*, **94**, 551 [1931–32]). This enzyme occurs in the kidney and intestine where normally no calcification takes place.

[23] H. D Kay, *Brit. J. Exptl. Path.*, **10**, 253 (1929); *J. Biol. Chem.*, **89**, 249 (1931); A. Bodansky, *ibid.*, **101**, 93 (1933).

[24] *Biochem. J.*, **29**, 2721 (1935); see also *ibid.*, **30**, 977 (1936).

less apatite and more carbonate than enamel. It also contains more magnesium.

TABLE LXIII

COMPOSITION OF ENAMEL AND DENTINE

	Dry Enamel (in per cent)	Dry Dentine (in per cent)
Ash	95.38	71.09
Nitrogen	0.156	3.43
Combined H_2O (calculated)	1.347	....
Combined CO_2	1.952	....
Calcium	37.07	27.79
Magnesium	0.464	0.835
Sodium	0.25	0.19
Potassium	0.05	0.07 or less
Phosphorus	17.22	13.81
Chlorine	0 3	Nil
Fluorine	0 025	0.0246
Silicon	0 003	....
Ca/P (Ca/P for apatite is 2.151)	2.153	2 012
Ca/Mg	79.89	33.29
P/CO_2	8.882	4.349
Ca : P : CO_2 (molar proportions)	10 : 5.994 : 0.479	10 : 6.412 : 1.036

Attention has been drawn to the so-called " anti-calcifying " action of certain cereals. E. Mellanby [25] thought the effect to be due to the presence of a specific decalcifying agent. It has been stated by M. Mellanby [26] that oatmeal and wheat germ, which experimentally produce the worst-calcified teeth, have far more calcium and phosphorus than other cereals which do not exert as marked an effect. However, Bruce and Callow [27] pointed out that much of the phosphorus in cereals is present in the form of inositolphosphate, which is not absorbed and therefore unavailable to the needs of the body. Lowe and Steenbock [28] have shown that, whereas maize is rachitogenic, it acquires antirachitic properties after treatment with hydrochloric acid. This is apparently related to the hydrolysis of phytin (calcium-magnesium salt of inositolphosphoric acid) and the liberation of utilizable inorganic phosphate. Phytin itself is a poorly available source of phosphorus when fed to the rat.

It may also be observed that cereals leave an acid residue. That this may be a factor contributing to decalcification or faulty bone formation, especially in the young, on diets inadequate with respect to calcium, is a possibility which, however, requires experimental verifica-

[25] *Brit. Med. J.*, **1**, 831 (1922); **2**, 849 (1922); **1**, 895 (1924); **1**, 515 (1926); H. N. Green and Mellanby, *Biochem. J.*, **22**, 102 (1928).
[26] *Physiol. Rev.*, **8**, 545 (1928).
[27] *Brit. Med. J.*, **2**, 172 (1932); *Biochem. J.*, **28**, 517 (1934).
[28] *Biochem. J.*, **30**, 1126 (1936).

tion. It has been clearly demonstrated that the administration of ammonium chloride, an acid-former, to dogs produces decalcification of bone, this being most conspicuous in young animals kept on a calcium-low diet.[29]

Dental Caries. It is impossible to dwell here at length on the controversial and intricate problem of the etiology of dental caries. There are apparently factors besides diet which are responsible for this condition. However, it has been brought out that diets which contain an abundance of protective foods (calcium, phosphorus, vitamins C, D, etc.) and which therefore promote normal calcification of the skeleton and a good state of nutrition generally are also the most effective in preventing the origin and development of dental caries. The subject has been reviewed recently by Cowell.[30]

The effect of fluoride in producing mottled enamel has been discussed elsewhere (p. 555).

The work of Schour and others [31] on the histology of the tooth and particularly of the enamel and dentine has revealed that incremental lines are laid down according to a well-defined rate and in a well-defined pattern. In the microscopic structure of the enamel and dentine are reflected with a high degree of accuracy and sensitivity the fluctuations in calcium and phosphorus metabolism which may have occurred in the period of tooth development.

The Skin. Of the two principal layers which compose the skin, the lower layer, the *dermis*, or *corium*, is vascular, and the upper layer, or *epidermis*, is avascular. The epidermis, in turn, may be said to consist of four layers or strata, the deepest of which derives considerable nourishment from the blood vessels and lymphatics of the corium. This is the *stratum germinativum*, in which cells are continually formed and are displaced toward the surface of the skin, the cells forming successively the other three layers, the strata *granulosum*, *lucidum*, and *corneum*. These cells, by the time they form the stratum corneum, are essentially dead and are eventually lost by desquamation. In these transitions important chemical changes are involved, our knowledge of which is unfortunately far from being complete. The cells of the stratum germinativum are metabolically active, one index of this being the relatively high concentration of a substance, presumably glutathione, which gives the sulfhydryl (SH) group reaction. The water content which is greatest in the lowest layer of the epidermis diminishes in the upper layers, as the surface is approached. Granules of an albuminoid, called *keratohyalin*, are scattered irregularly in the stratum germinativum and are very abundant in the stratum granulosum. These granules fuse together

[29] H. L. Jaffe, A. Bodansky, and J. P. Chandler, *J. Exptl. Med.*, **56**, 823 (1932).
[30] *Nutrition Abstracts & Revs.*, **5**, 567 (1936).
[31] For references to the literature, the reader is referred to an editorial in the *J. Am. Med. Assoc.*, **108**, 807 (1937). See also I. Schour, S. B. Chandler, and W. R. Tweedy, *Am. J. Pathol.*, **13**, 945, 971 (1937).

in the stratum lucidum and undergo still more profound change in the stratum corneum, where the characteristic properties of the keratohyalin are lost and keratin is formed.[32]

Keratin is an albuminoid and is the chief constituent not only of the epidermis but of its derivatives, including the hair, nails, hoofs, horns, feathers, tortoise shells, and the shell membrane of bird's eggs. Since the keratins from various sources differ somewhat in composition, it is to be assumed that there is not one keratin but a group of these albuminoids. Indeed, there is a strong probability that even in the same source there may be more than one keratin. A distinction has been made between the so-called keratin " A," which is so resistant that it is even insoluble in fuming nitric acid and in a mixture of sulfuric acid and hydrogen peroxide, and keratin " B," which is soluble in these reagents. The keratins are insoluble in the usual protein solvents and are not acted on by pepsin and trypsin. They give positive xanthoproteic and Millon's reactions.

According to Wilkerson's[33] analyses, human epidermis (stratum corneum) contains 2.31 per cent cystine, 0.59 per cent histidine, 3.08 per cent lysine, and 10.01 per cent arginine. The molecular ratio of histidine, lysine, and arginine was found to be 1 : 5.6 : 15.1, which is in good agreement with the analytical values that have been obtained for the keratins of human hair and finger nails.

A second important constituent of the skin, present especially in the deeper layers of the epidermis, is the pigment melanin, the occurrence of which has been discussed in an earlier chapter (p. 400). In small aggregates melanin appears yellowish brown in color, but more dense masses appear black. It is present in larger amounts in negroes than in Caucasians. Melanin is deposited in the skin when one is sunburned. Besides its occurrence in the skin of man and animals, it is normally present as the pigment of the hair and the choroid of the eye. It is also found in many low forms of life, as in the black secretion of the squid.

The formation of melanin from its precursors is brought about by an enzyme, the existence of which in the epidermal melanoblasts of the skin has been demonstrated by Bloch.[34] This enzyme has been shown to produce melanin from 3 : 4 dioxyphenylalanine. The failure of melanin formation in albinos has been associated with the absence of this enzyme.[35] Copper may be of importance in the enzymic reactions

[32] For a more detailed account, consult E. V. Cowdry, "The Skin and Its Derivatives," in Cowdry's "Special Cytology," 2d edition (1932), Vol. I.

[33] *J. Biol. Chem.*, **107**, 377 (1934); **112**, 329 (1935–36).

[34] *Z. physiol. Chem.*, **98**, 226 (1917). This enzyme was named *dopa* oxidase by Bloch, the term being derived from the initial letters of the name of its substrate, *di-oxy-phenyl-alanine*. The action of this enzyme is not limited, however, to the conversion of only this substrate into melanin. Compare with the work of Raper on tyrosinase, *Physiol. Rev.*, **8**, 245 (1928).

[35] Garrod describes albinism as an inborn error of metabolism. For a description

involved in the production of pigment. Depigmentation of fur has been observed in black or "hooded" rats and other animals kept on a copper-free diet. This defect was specifically cured by copper.[36]

In addition to melanin, the presence of a lipochrome has been described in skin and hair, to which is attributed the characteristic red coloration which is often seen in hair.

All layers of the epidermis contain fatty substances, approximately one-fifth of which, according to Eckstein's analyses,[37] is free cholesterol. It, as well as the phospholipid fraction, is present in the skin in much greater proportion than in subcutaneous fat. The skin lipids contain a considerable amount of free fatty acid. Associated with cholesterol in the skin, as elsewhere, is at least one other sterol, which on irradiation acquires antirachitic potency. As has been described in the preceding chapter, this is the probable explanation for the beneficial effect of exposure to sunshine.

The skin contains a wide variety of other organic and inorganic substances, including glucose, glycogen, and mucin, sodium, potassium, calcium, magnesium, iron, silicon, arsenic, in traces, etc. Individuals that have been exposed to certain toxic metals, such as nickel, silver, gold, or lead, contain these in their skin. The sebaceous glands produce a waxy secretion, called sebum. The perspiration is formed by the sweat glands and contains among its constituents many substances which are also found in the urine.

Muscle. Skeletal muscle contains approximately 75 per cent water and 25 per cent solids. Of the latter about 20 per cent is protein, the remaining 5 per cent consisting of lipoids, carbohydrate, inorganic salts, and the so-called extractives. Plain muscle has a somewhat higher water content (80 per cent) than striated muscle and contains more nucleoprotein and less creatine. Muscle is known to contain four kinds of protein, apart from those of the connective tissue and stroma: myosin, myogen, globulin *X* and myoalbumin. Myosin, which is a globulin, has been studied in great detail by Weber,[38] Edsall [39] and others. According to different analyses, it comprises from about 50 to 70 per cent of the

of the nature of this condition, the student is referred to A. E. Garrod, "Inborn Errors of Metabolism," 2d edition, Oxford Univ. Press (1923).

The pathological occurrence of melanin is discussed by H. G. Wells in his "Chemical Pathology," Saunders, Philadelphia (1925), Chapter XX.

[36] H. L Keil and V. E. Nelson, *J. Biol. Chem.*, **93**, 49 (1931); F. J. Gorter, *Nature*, **136**, 185 (1935).

Black skin and hair have been found to contain more copper than brown skin and hair and considerably more than white skin and hair. U. Sarata, *Japanese J. Med. Sci.*, II *Biochemistry*, **3**, 79 (1935). See also I. J. Cunningham, *Biochem. J.*, **25**, 1267 (1931).

[37] *J. Biol. Chem.*, **69**, 181 (1926).

[38] *Ergeb. Physiol.*, **36**, 109 (1934).

[39] *J. Biol. Chem.*, **89**, 289 (1930); A. L. von Muralt and J. T. Edsall, *ibid.*, **89**, 315, 351 (1930).

total intracellular protein fraction. Next in quantitative importance is globulin *X* (22.5 per cent, according to Smith,[40] 26.5 per cent, according to Weber) and myogen (9 per cent, Smith; 26.5 per cent, Weber [38]). Myoalbumin is about 1 per cent of the total.

Undenatured myosin and globulin *X* are insoluble in water, but soluble in dilute salt solutions, whereas myogen and myoalbumin are water-soluble. The individual proteins are also characterized by their isoelectric points: myosin *p*H 5.5, globulin *X* *p*H 5.2, myogen *p*H 6.7, myoalbumin *p*H 3.3. Bailey[41] has pointed out certain distinctions in the amino-acid constitution of myosin and myogen as shown by the following data (in per cent):

	Tyrosine	Tryptophane	Cystine	Methionine
Myosin (rabbit muscle).....	3.40	0.82	0.53	3.4
Myogen (" ").....	4.21	1.51	1.80	2.8

Among the properties of myosin are its high degree of hydration, the marked viscosity of its solutions, and the tendency to form gels. It is stated that even concentrated precipitates of myosin contain as much as 98 per cent water, a property which probably has a bearing on the ability of living muscle to withstand dehydration. Myosin shows double refraction or anisotropism and possesses other physical properties which suggest its relation to the rod-like structural elements of the anisotropic disks in muscle. Muscle globulin is probably the material of which the fibrillae are composed.

Muscle hemoglobin (myohemoglobin, myoglobin) has been isolated in crystalline form [42] and has also been characterized with respect to molecular weight (35,000), isoelectric point (*p*H 6.99), and absorption spectrum. These distinguish it from blood hemoglobin. The iron content, however, is the same in both compounds.

Boiling water extracts from muscle both inorganic salts and a variety of organic compounds, the latter being termed " extractives." Among the nitrogenous organic extractives are included creatine, creatine-phosphate, creatinine, inosinic acid, adenylic acid, adenosinetriphosphoric acid (adenylpyrophosphoric acid), glutathione, various purines, such as hypoxanthine, etc. These have been described in other connections. The amount of creatine in human skeletal muscle is about 350 to 400 mg. per 100 grams, and in smooth muscle (such as that of the human uterus) about one-fifth as much. About 5 to 10 mg. of creatinine per 100 grams is present in striated muscle and somewhat smaller quantities in smooth muscle. The nitrogenous base carnosine, $C_9H_{14}N_4O_3$, has been isolated from meat extracts. On hydrolysis

[40] *Proc. Roy. Soc.* (*London*), *B*, **124**, 136 (1937).

[41] *Biochem. J.*, **31**, 1406 (1937).

[42] A. H. T. Theorell, *Biochem. Z.*, **252**, 1 (1932); see also G. B. Ray and G. H. Paff, *Am. J. Physiol.*, **94**, 521 (1930); M. N. J. Dirken and H. W. Mook, *J. Physiol.*, **69**, 210 (1930).

it yields histidine and β-alanine. Anserine, first isolated from goose muscle, is methyl carnosine (β-alanylmethylhistidine). Another base is carnitine, $C_7H_{15}NO_3$, which is a derivative of betaine. The presence of acetylcholine is of special interest (p. 634). The non-nitrogenous organic extractives include glycogen, glucose, the hexahydric alcohol, inosite, or inositol, $C_6H_6(OH)_6$, the various hexosephosphates described in the discussion of carbohydrate metabolism, *l*-lactic, and other intermediates.[43] Mention has been made elsewhere (p. 373) of the occurrence and significance of phospholipids and cholesterol in muscle.

The inorganic constituents (found in the ash) of striated muscle include potassium (0.25 to 0.4 per cent), sodium (0.06 to 0.16 per cent), magnesium (0.02 to 0.03 per cent), chloride (0.04 to 0.08 per cent), sulfur (0.19 to 0.23 per cent), and phosphorus (0.17 to 0.25 per cent).[44] The calcium content is normally about 70 mg. per 100 grams (0.07 per cent). It is considerably reduced in rickets.[45] Practically all the sulfur is present in organic combination in protein. In striated muscle about 80 per cent of the phosphorus is inorganic and the remainder organic. The relation is very different in smooth muscle, where the inorganic phosphorus is frequently 40 per cent or less (as in uterine muscle) and the organic phosphorus about 60 per cent. The buffering power of smooth muscle is said to be less than that of striated muscle.

More sodium chloride and less potassium chloride are present in plain muscle than in striated muscle. The ratio of sodium to potassium in the former is 1 : 1.5, whereas in the latter it is 1 : 5.[45]

Brain and Nerve.[46] The adult human brain contains about 77 per cent of water; the gray substance contains 84 per cent and the white substance 70 per cent. The protein content is 8–9 per cent. Block[47] has recently summarized our present inadequate knowledge of the chemistry of the neuroproteins. He also analyzed the brain proteins of man and of certain animals (monkey, beef, sheep, rat, guinea pig) and found them to be very similar as regards the proportional distribution of the amino acids histidine, lysine, arginine, tryptophane, and tyrosine.

[43] For a fuller account the reader is referred to D. W. Wilson's review "Nitrogenous Muscle Extractives," *Yale J. Biol. Med.*, **4**, 627 (1932). See also F. Kutscher and D. Ackermann's article on the comparative biochemistry of vertebrates and invertebrates. *Ann. Rev. Biochem.*, **2**, 355 (1933); **5**, 453 (1936).

[44] E. B. Meigs and L. A. Ryan, *J. Biol. Chem.*, **11**, 401 (1912). See also series of papers on the exchange of water between muscle and blood, A. B. Hastings and L. Eichelberger, *J. Biol. Chem.*, **117**, 73 (1937); Eichelberger and Hastings, *ibid.*, **118**, 197, 205 (1937); Eichelberger, *ibid.*, **122**, 323 (1938).

[45] The reader is referred to the following reviews: D. M. Needham, "Red and White Muscle," *Physiol. Rev.*, **6**, 1 (1926); *Ann. Rev. Biochem.*, **6**, 395 (1937); C. L. Evans, "Physiology of Plain Muscle," *Physiol. Rev.*, **6**, 358 (1926); J. K. Parnas, "The Chemistry of Muscle," *Ann. Rev. Biochem.*, **1**, 431 (1932); **2**, 317 (1933); W. O. Fenn, "Electrolytes in Muscle," *Physiol. Rev.*, **16**, 450 (1936).

[46] For a review of present knowledge the reader is referred to the recently published book by I. H. Page, "Chemistry of the Brain," C. C. Thomas, 1937.

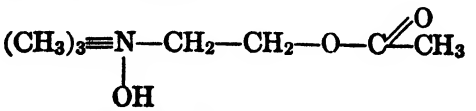
[47] *Yale J. Biol. Med.*, **9**, 445 (1937); *J. Biol. Chem.*, **119**, 765 (1937).

A substance possessing some of the properties of keratin (low solubility and resistance to digestive enzymes) was prepared from brain by Ewald and Kühne [48] in 1877. Gray matter (and cerebellum) contains about 0.3 per cent, white matter about 1.1 per cent, the corpus callosum 2.5 per cent, and sciatic nerve 0.6 per cent. Block [49] has shown that neurokeratin contains histidine, lysine, and arginine in the proportions of 1 : 2 : 2. It differs therefore from the true keratins, which contain these amino acids in the proportions of 1 : 4 : 12.

The total lipids comprise 12–15 per cent of the weight of the fresh adult brain and are distributed approximately as follows: phosphatides 5.7–6.8 per cent, cerebrosides 1.3–2.6 per cent, sulfatides 0.66–1.8 per cent, cholesterol 3.63–4.8 per cent. The organic extractives (creatine, phosphocreatine, amino acids, etc.) amount to 1.1 to 2.0 per cent, and the inorganic extractives to about 1 per cent. The ash content of whole fresh brain is approximately 1.5 per cent; the gray matter yields 1.0 per cent ash and the white matter 1.75 per cent. Potassium represents 34.8 per cent of the total ash of gray matter; phosphorus 24.1, sodium 20.8, chlorine 11.4, sulfur 5.6, magnesium 1.9, calcium 1.0, and iron 0.6 per cent.

The spinal cord contains a greater proportion of unsaturated phospholipids than any other part of the central nervous system. The water content is 74 per cent, and the total amount of lipids is about 18 per cent. The peripheral nerves, on the other hand, contain only about 60 per cent water. Medullated fibers have more cerebrosides than phospholipids, whereas the reverse relationship is present in the non-medullated fibers. Approximately the same amount of neurokeratin is contained in peripheral nerves as in the gray substance of the brain.

Among the substances involved in the transmission of nerve impulses, acetylcholine is of particular interest. In 1921 Otto Loewi [50] demonstrated that stimulation of the vagus nerve (frog) caused the liberation of an agent which was evidently responsible for the well-known cardio-inhibitory effect. The compound was later shown to be acetylcholine. Ordinarily, acetylcholine does not accumulate in tissues, owing to its rapid hydrolysis by the enzyme *choline esterase*, the action of which may be inhibited, however, by eserine. The presence of acetylcholine has been demonstrated in brain, spinal cord, peripheral nerves, spleen, blood, muscle, and other tissues.



Acetylcholine

[48] *Verh. med. Naturwiss.* (*Heidelberg*), **1**, 357 (1877).
[49] *J. Biol. Chem.*, **94**, 647 (1931–32).
[50] *Arch. ges. Physiol.*, **89**, 239 (1921); see also *Harvey Lectures*, **28**, 218 (1932–33); *Proc. Roy. Soc.* (*London*), *B*, **118**, 229 (1935).

Acetylcholine reproduces the peripheral effects obtained when parasympathetic nerves are stimulated. This is often described as the "muscarine" effect. It may be abolished by atropine. Acetylcholine also stimulates the cells of the sympathetic ganglia, an effect that has been referred to as the "nicotine" action. It is not antagonized by atropine, but may be paralyzed by large amounts of nicotine. The peripheral effects of the parasympathetic nerves, as well as the transmission of impulses from the preganglionic fibers across the synapses in the sympathetic ganglia, are mediated by acetylcholine. It has been shown, more recently, that acetylcholine is also liberated at motor nerve endings in voluntary muscle and that this occurs even after autonomic and sensory innervation are eliminated.[51] From this it is seen that the action of acetylcholine is not restricted to the autonomic system but is of general importance. It should also be observed that besides acetylcholine there is an adrenal-like substance (sympathin),[52] and possibly other agents, which are active in the chemical transmission of nerve impulses.

[51] H. H. Dale, W. Feldberg, and M. Vogt, *J. Physiol.*, **86**, 353; 87, 394 (1936); see also H. H. Dale, *Brit. Med. J.*, **2**, 1161 (1934).

[52] W. B. Cannon, *Am. J. Med. Sci.*, **188**, 144 (1934); W. B. Cannon and A. Rosenblueth, *Am. J. Physiol.*, **112**, 268; **113**, 251 (1935).

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