THE BASIS OF CHEMOTHERAPY

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TO

THE LATE

GEORGE BARGER

TEACHER AND FRIEND

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PROLOGUE

CHEMOTHERAPY, as its name implies, is a hybrid subject. Its boundaries stretch from organic chemistry, through biochemistry and physical chemistry to bacteriology, pharmacology and therapeutics. Because of the breadth of its field, we considered a book on chemotherapy covering several of these aspects might be a useful contribution to a rapidly developing subject. Although therapeutic use of any chemical might be regarded as chemotherapy, it is usual, in practice, to restrict the meaning of the term to the chemical treatment of diseases of microbial origin with a view to eliminating the microbial infection. Such a restriction has been adhered to in this book.

We have attempted to weld many diverse sciences into a single framework in order to find a basis for chemotherapy. In so doing, we must inevitably cover ground familiar to specialists in each field. The trained organic chemist may regard our exposition of chemical theory as superfluous, the expert microbiologist may consider that we have included unnecessary fundamentals of bacteriology, while the specialist in biochemistry may feel that we have wasted space in a detailed account of enzymology and cell physiology. It has been our aim, however, to provide a sufficient background to each subject so that the student of chemotherapy may be lured into the study of subjects outside his own original field.

The history of chemotherapy during the last half century is largely one of painstaking development by the organic chemist of chance observations made by the experimental pathologist or the microbiologist. Only those who have played a part in this development can realise the immense effort that has gone towards the perfection of clinically useful drugs. In seeking a theoretical basis for our subject, we may seem to have done less than justice to this aspect, but it is already covered by standard works. The theoretical developments with which we are concerned provide no royal road to

the production of new chemotherapeutic drugs, but they begin to provide a rationale for the whole subject. If this book does something to focus attention upon the modes of action of drugs rather than upon the synthesis of ever more variants on their chemical structure, it will, to some extent, have fulfilled its purpose.

We ask the indulgence of those more expert than ourselves ; we are amateur authors with a limited fund of time and energy available after full days at the laboratory bench. We should be grateful, therefore, if our colleagues would draw our attention to any obscurities or inaccuracies so that we may subsequently rectify them.

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The Microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth ;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of forty separate bands ;
His eyebrows of a tender green ;
All these have never yet been seen –
But Scientists, who ought to know,
Assure us that they must be so . . .
Oh ! let us never, never doubt
What nobody is sure about.

H. Belloc.

CHAPTER I

HISTORICAL INTRODUCTION

Traditional remedies

THE art of chemotherapy is as old as civilisation; the science of chemotherapy is the child of to-day. Treatment of disease has always been associated with administration of medicines; while many of these were worthless and overlaid with superstition and magic, others were of real value, recognised no doubt by acute observation following a process of trial and error. To-day a more scientific basis for the use of certain of these medicines has gradually developed through the systematic isolation and testing of their active principles.

In the East, particularly in China, herbal remedies have been in use for more than five thousand years. About 3000 B.C. the Emperor Sheng Nung recorded many of the known remedies in the Book of Herbs. Even now, the curative value of many of these has not been scientifically investigated, but in some cases the active principles have been isolated and their therapeutic value established. The plant *Ma Huang*, of the ephedra species, was noted by Sheng Nung to be a diaphoretic and circulatory stimulant. In 1887 the alkaloid ephedrine was isolated from the plant by Nagai, but its therapeutic value was not recognised until after 1923, when Chen showed that a decoction made from *Ma Huang* produced an effect on blood pressure similar to that of adrenaline (Chen and Schmidt, 1923). The drug *Ch'ang Shang* was also included in the Book of Herbs where it was stated to be valuable against malaria paroxysms and similar fevers. The antimalarial action of an extract of *Ch'ang Shang* has recently been confirmed and the active alkaloid identified (Tonkin and Work, 1945; Koepfli, Mead and Brockman, 1947).

Many other traditional remedies have yielded active principles which are still in use to-day. The fruit of the *Kalaw* or chaulmoogra tree was used by the ancient Hindus for the

cure of leprosy. An oil from the seed, chaulmoogra oil, was introduced into Western medicine in 1854 by Mourat, and is still used extensively in the treatment of leprosy. The anthelminthic, oil of chenopodium, from the tree *Chenopodium anthelminthicum*, was probably used by the Aztecs as well as by Eastern civilisations, as is indicated by the two names, Jerusalem Oak and Mexican Tea. Male fern was esteemed by the Greeks and recommended by Theophrastus, Pliny and Galen ; while the plant *Artemisia maritima*, which has yielded the anthelminthic drug santonin, was known to Greek, Roman and Arabic medicine. Ipecacuanha root was introduced in 1658 by Guillaume le Pois into Europe from Brazil, where its medicinal qualities in curing diarrhœas were known to the natives. The root is also said to be an ancient Indian and Chinese cure for chronic dysentery. An alkaloid, emetine, isolated from ipecacuanha by Pelletier and Magendie in 1817, was shown by Tull Walsh in 1891 to be active against amœbic dysentery.

Other valuable plant products were known to the ancient civilisations of South America. The Spanish conquest of these countries in the sixteenth century led to the introduction into Europe of several drugs believed to have been in use by the natives before conquest. Cinchona bark, originally known as Jesuit bark, was brought to Spain in 1633 by the Jesuits, who are supposed to have been told by Peruvian natives of its curative properties for fevers. The first record of its use has been found in the writings of an Augustinian monk, Antonio de la Calancha, who stated in 1633 that the bark of the " fever tree " cures " the fevers and tertianas ; it has produced miraculous results in Lima." Tradition tells that the Countess of Chincon, wife of the Viceroy of Peru, was cured dramatically of a malarial fever in about 1630 ; also that the name " Cinchona," introduced by Linnæus for the plant genus, was suggested by her title. Recent research (Haggis, 1941) has, however, shown that this particular lady died before her husband went to Peru, and that the second wife never contracted a fever during her husband's stay there. The Viceroy himself was much troubled

with fevers which were treated by traditional bleeding methods but he was never cured by administration of any kind of drug. The alkaloid quinine, which was isolated in 1820 from cinchona bark by Pelletier and Caventou, has been, up till recently, the most effective cure known for malaria.

Growth of European medicine

European medicine may be said to have been founded by Hippocrates in the fourth century B.C. Later, Galen (A.D. 131-200) established a formal system of medicine which persisted practically unchanged for 1500 years. Galen believed that a state of bodily health was preserved by the presence in their proper proportions of the four humours—heat, cold, dryness and moisture. Disease was supposed to result from a disturbance of the balance of these humours and could be cured by administration of various drugs possessing these fundamental qualities. Galen rejected the use of simple metallic remedies such as mercury, which seem to have been used at that time, and introduced a complicated system of therapeutics based upon herbal remedies.

Bound by Galen's formidable mixtures, European medicine suffered a long period of stagnation which was not relieved until the sixteenth century when a fresh spirit of inquiry became evident. Galen's doctrines were largely modified by the teachings of Paracelsus (Theophrastus von Hohenheim, born 1493). Much of Paracelsus' medical theory was overlaid with a nonsensical mass of astrology, mysticism and alchemy, but he ridiculed the absurd mixtures of herbs advocated by Galen's followers, and substituted some simple and powerful remedies, many of them metallic. He believed that there was a specific remedy for each disease, and was able to contribute one specific to medicine—namely, mercury for syphilis. This cure had been in use sporadically for some time, and its successful application by Paracelsus did much to popularise his other mineral cures, such as antimony.

Unfortunately these metallic medicines (tartar emetic, calomel, etc.) became over-popular. They are mostly highly poisonous, and it is probable that over-dosage with this type

of drug was responsible for much additional sickness and suffering. Antimony, in particular, seems to have enjoyed much popularity in the sixteenth century. The metal was used to make goblets in which wine was allowed to stand until it had acquired emetic properties, and everlasting pills of the metal were administered and recovered for future use after they had fulfilled their function. In 1566 the doctors of Paris decided, not without considerable opposition, that antimony was a poison, not a remedy, and its use was banned by Act of Parliament. However, in 1657, Louis XIV was treated with antimony for typhoid fever, and, as a consequence of his recovery, antimony was restored to the pharmacopœia.

In addition to metallic remedies, European medicine boasted, until fairly recently, a strange array of drugs; pearls, musk, crocodile dung, unicorn's horn, Egyptian mummy, sarsaparilla and many other strange substances were used in decoctions. The moss scraped from the skull of a criminal who had hung in chains, known as "usnea," was endowed with remarkable curative properties; it was an official drug in the pharmacopœia until the nineteenth century.

Although the seventeenth century saw the Spanish introduction into Europe of cinchona bark as an antipyretic and also of ipecacuanha, conservative medical groups viewed the new drugs with suspicion because their use did not conform to the teachings of Galen. Others looked upon cinchona with equal suspicion because the Jesuits sold it. The first official recognition came in 1677 when cinchona bark was included in the London Pharmacopœia under the title "*Cortex Peruanus*." Ipecacuanha evidently obtained popularity as a cure-all, for it is included in the famous diaphoretic "Dover powders" compounded by the buccaneer physician, Dr Dover (1660-1742).

Germ theory of disease

By the beginning of the nineteenth century, a certain amount of scientific method was being introduced into medicine, and doctors were learning to assess by experiment

whether a drug was of value in curing a certain disease. This led to elimination of many useless drugs, but not to introduction of new ones. For this to take place on anything except an empirical basis, it was first necessary for the cause of disease to be understood.

The germ theory of disease was not established until the middle or late nineteenth century. Its forerunner was a theory of the contagious nature of disease propounded in 1546 by Girolamo Fracastoro of Verona. At this time, disease was considered to be due either to divine displeasure or to elemental causes, such as comets, earthquakes, floods or changes in the air. Malaria, known as "shivering ague," was, for example, attributed to a nocturnal "miasm." Fracastoro explained contagion by postulating *the existence of seeds of disease which were capable of being propagated from one individual to another*. Propagation occurred by direct contact, by contact with fomites (objects which conserve the seeds), or at a distance. He gave accurate descriptions of Italian epidemics of typhus, plague, rabies and syphilis, and produced a valuable account of the general therapy of contagions. His work was held in the highest repute during his lifetime, but by the end of the sixteenth century it had been forgotten, and all that Fracastoro had achieved had to be rediscovered in the nineteenth century.

The first proof that disease of any kind is associated with living micro-organisms resulted from work on silk-worms, published by Agostino Bassi of Lodi in 1835. He demonstrated the existence of transmissible pathogenic micro-organisms which caused the silk-worm disease *mal del segno*, and propounded a theory of contagion in such human diseases as syphilis, variola, typhus, plague and cholera. He also wrote on the destruction of germs by heat and chemicals. Study of anthrax by various investigators, notably Davaine, between 1850 and 1865, provided the first scientific proof of the association of disease in animals with micro-organisms. The work concluded with a statement by Davaine that bacteria are the cause of anthrax. This was confirmed and firmly established by Pasteur, who, after successive subculture of

the organism, produced a culture which still infected susceptible animals. This technique, introduced by Pasteur, enabled him to show that any non-viable material carried into the primary culture from blood or tissues would be too highly diluted during subculture to be responsible for the infective nature of the final culture.

At this time Pasteur also established that yeasts are the causative agents of fermentation in wine and other organic solutions. In 1861 he published his work disproving the theory of spontaneous generation of micro-organisms, a favourite subject of controversy among biologists. He also developed methods for the isolation and cultivation of bacteria, for the study of their effects on animals, and for the passage of a virus disease, such as rabies, through an animal.

In 1865 Lister successfully applied the principles of Pasteur's germ theory to surgery, and established that, by cleanliness and sterilisation of wounds with phenol, the dreaded and all too common putrefaction of wounds could be prevented. The success achieved by Lister in the development of his antiseptic system of surgery did much to make the germ theory acceptable to the medical profession. Further work by Davaine in 1872 on septicæmia suggested that sepsis was bacterial in origin, since a rabbit could be killed by injection of an amount of blood from an infected rabbit corresponding to one-billionth of a drop. The question was finally proved by Koch in 1878 when he demonstrated that six different infective diseases could be induced by injection of " putrid " fluids into animals. Koch perfected bacteriological techniques introduced by Pasteur, and applied staining techniques and oil immersion to the microscopic study of bacteria. His first work in 1876 was a remarkably complete description of anthrax bacilli and spores, and their methods of dissemination in animals.

The establishment of the germ theory of disease and the perfection of bacteriological techniques opened up a new era in medicine. Advances in all fields were extremely rapid, and during the last twenty years of the nineteenth century micro-organisms responsible for many infective diseases were

isolated and characterised. Even the origins of protozoal diseases were recognised, the malaria parasite being described by Laveran in 1880. It was at this time that the science of chemotherapy was conceived. The thoughts of investigators in bacterial medicine naturally turned to methods of destroying bacteria by drugs after they had invaded the host, as well as by disinfectants outside the body. It is interesting that the first experiments on chemotherapeutic treatment should have been made by the investigator who did so much to establish bacteriology. In 1881 Koch infected guinea-pigs with anthrax bacilli, and treated them with subcutaneous injections of a solution of mercuric chloride, used because of its powerful *in-vitro* bactericidal action on micro-organisms. The animals died of anthrax in forty-eight hours, in spite of dosage, both before and after inoculation, of an amount of mercuric chloride sufficient to prevent all bacterial growth in broth.

This pioneer experiment showed at once one of the main difficulties still encountered to-day in chemotherapy: namely, substances which are highly active against micro-organisms *in vitro* are often ineffective *in vivo*. Even before Koch's experiment, an indication of this difficulty had appeared; Baxter (1875), using vaccine lymph and glanders nodules, showed that organic matter diminished the activity of disinfectants. We now know that many disinfectants are adsorbed by proteins, so that the uptake of disinfectant by the proteins of host blood and other cells diminishes the amount available to combat infecting organisms.

Effect of dyes on tissues and micro-organisms

The important subject of distribution of drugs in the body was continually stressed by Ehrlich, father of the science of chemotherapy. Ehrlich's first work was on the distribution of dyestuffs in the animal body (summarised, 1885). His interest in this field was aroused, when he was a student, by a paper on lead poisoning, by Heubel, claiming that organs in which lead accumulated could also fix the metal from solution after death. Ehrlich was led from this work to

believe "that the ways and means by which drugs are distributed over the body must be of the greatest importance in the rational development of therapeutics" (Ehrlich and Hata, 1911). Accordingly, he examined the distribution of dyestuffs, first in blood, and next in living animals. Dyestuffs were chosen because of the obvious ease in following their distribution. An early result of this work was the differentiation and classification of various types of body cells, particularly blood leucocytes. Following this, Ehrlich carried out his well-known experiments on vital staining. On killing an animal some time after injection of methylene blue, he found that the only tissues dyed were those of the nervous system, all the nerves being sharply defined along their whole length. By the use of many different dyes, he found that certain dyes stained specifically certain organs or types of cells, while others were fairly general in their action. For example, many basic dyes such as Bismark brown, neutral red, flavanilin or methylene blue stained nerve tissue only, while only one acidic dye, alizarin, had this property. These so-called "neurotropic" dyes lost this property on conversion to sulphonic acid derivatives. These facts were explained by Ehrlich by assuming that acidic dyes are bound in the blood by the alkali present, while basic dyes are not held in blood by any chemical affinities and are thus freer to diffuse into surrounding tissues. He emphasised that similar differences in distribution of colourless substances in the body are likely to occur.

Other interesting results of the study of vital staining were series of experiments on the oxygen requirements of organs. After injecting either alizarin blue or indophenol in the colloidal state into the circulation of an animal, Ehrlich found, on killing the animal, that some organs were coloured blue, while other organs had reduced the dye and contained the colourless leuco product. Organs which ordinarily did not reduce the dye, did so when a state of asphyxia was established. From this work he deduced the relative combining powers for oxygen of different tissues. The results were explained on the supposition that in cell protoplasm side-chains existed whose function was oxygen fixation; the affinity for

oxygen of these side-chains was assumed to vary in different organs. Here we find the germ of Ehrlich's side-chain theory on which he later based his chemotherapeutic theories.

It was natural at a time when new developments were continually being made in bacteriology, that Ehrlich should turn his attention to staining of bacteria. In 1881 he found that bacteria as well as tissues were stained by methylene blue. In 1882 he developed an acid-fast stain for tubercle bacillus which is the essence of the Ziehl-Neelsen staining method used to-day, and which consists of an aniline-water solution of methyl violet or other aniline dye, with vesuvin or methylene blue as a counter-stain. This aniline-water methyl violet mixture of Ehrlich was also used by Christian Gram (1884) in the now universally known "Gram stain." The Gram process of staining has divided bacteria into two groups, Gram-negative and Gram-positive, according to whether they retain the aniline dye after suitable treatment. This grouping is also found to divide bacteria according to their susceptibilities to the action of bacteriostatic agents, basic dyes, detergents and antibiotics. Gram-positive organisms are attacked under normal conditions by such agents. Gram-negative organisms are frequently more resistant, but are more susceptible to enzymic digestion and to lysis by immune serum in the presence of complement. Henry and Stacey (1943, 1946) showed that the fundamental difference between Gram-positive and Gram-negative organisms is related to the presence in Gram-positive organisms of a magnesium ribonucleate which can be separated from the "cytoskeleton" by suitable extraction methods. The "cytoskeleton," which is Gram-negative, can be recombined with magnesium ribonucleate and then stains Gram-positive once more.

The bactericidal action of dyes was early recognised. Rozsahegyi (1887) clearly demonstrated that various species of bacteria do not develop on nutrient gelatin in the presence of dyes such as carmine, methylene blue, gentian violet, vesuvin, fuchsin or methyl violet, and pointed out that certain dyes possess a selective action against certain micro-organisms. The potent action of aniline dyes on bacteria was

recorded by Stilling (1890), who obtained inhibition of growth of certain bacteria with dilutions of dye of 1 in 2,000,000. He concluded that aniline dyes, in sufficient concentration, will prevent the development of practically all bacteria. Stilling also propounded a theory for the mode of action of dyes which is interesting in the light of modern conceptions of germicidal action. He observed that when micro-organisms were first placed in solutions of dyes, the dye was deposited in the intermicellary spaces of the covering membrane, which was thereby stained ; from these it could be extracted merely by placing the cell in water. This occlusion in intermicellary spaces, even without chemical combination, was considered to be sufficient to affect the metabolism of the cell so as to cause serious consequences to the organism, but not its death. On longer exposure, or in the presence of a greater concentration of dye, a certain amount of dye penetrated into the cell protoplasm where it may have existed as in the cell membrane, and from which it could also be extracted with water. Death of the cell resulted only when still longer exposure or still higher concentration of dye caused increased storage in the protoplasm, in consequence of which "the vital movements of the plasmatic micellæ are arrested." In this case the dye could no longer be extracted with water. This work was further extended by experiments on infections in animals, and Stilling recommended that a mixture of blue dyes known as "pyoctanin" should be used for the treatment of minor surgical infections, as it was non-toxic to animal tissues.

In 1891 Ehrlich performed his first experiment in chemotherapy. He found that methylene blue stained malaria parasites very effectively, a result which induced him, in collaboration with Guttmann, to try the dye on malarial patients. Some success was obtained : cases of tertian malaria were cured by administration of methylene blue, but the results were not sufficiently impressive to warrant the substitution of methylene blue for quinine. We shall see later, however, that this work played a guiding part in the development of the antimalarial drugs plasmoquine and atebrin.

Knowledge of disease at the beginning of the twentieth century

Ehrlich started his intensive work on chemotherapy in 1902. Before considering this work, it may be helpful to review the extent of knowledge at that time regarding cure and prevention of disease. The causative agents of most infective diseases, with the exception of virus diseases, were by then known. Experimental transmission of bacterial infection in laboratory animals was possible, as was the isolation and maintenance of pure strains of bacteria. The bactericidal action *in vitro* of phenolic compounds and dyes was known, and a fairly reliable method of standardising bactericidal action was achieved in the Rideal-Walker test (Rideal and Walker, 1903). Bacterial immunity was well known and serum therapy was established as a means of curing disease.

Ehrlich himself had made a major contribution to immunological work, which culminated in his theory of immunity published in 1897. He continually emphasised the chemical side of immunology, seeking to prove that the interaction between toxin and antitoxin was a chemical one and not, as was held by others (Bordet, 1903), due to physical forces. We have already mentioned that Ehrlich considered protoplasm to contain a grouping responsible for the fixation of oxygen. He also believed that protoplasm contained numerous other "receptors" whose normal function was to anchor by chemical combination a variety of foodstuffs as a preliminary step to their incorporation into the cell substance. The theory of immunity was an extension of this idea. The toxins (or antigens) combined with the specific receptors in the cell, just as the food molecules were supposed to do; but since the toxins were foreign substances not concerned in the normal economy of the cell, the receptors in question were diverted from their normal function. The cell was stimulated to replace these useless receptors, and in making new ones, formed an excess which was finally thrust off into the blood. The excess receptors in the blood constituted the specific antitoxin or antibody. This theory, now known to have many failings, had the merit that it kept to the fore the important

point that chemical specificity is an essential feature of immunological reactions. It also formed a basis for Ehrlich's views on chemotherapy.

The position with regard to diseases of protozoal origin was different. Although the causative agents of these diseases were known, there were no methods of cultivating the micro-organisms *in vitro*. A technique for transmitting trypanosome infections through a series of mice, developed by Laveran and Mesnil (1902), enabled stable strains of trypanosomes to be maintained *in vivo*. Many protozoal infections do not easily give rise to antibody formation, and are therefore not amenable to immunological methods of treatment. However, some of the traditional remedies of medicine were fairly effective in combating certain of the protozoal infections: malaria was cured by quinine, and amœbic dysentery by emetine or ipecacuanha. Some indications of the possible effectiveness of arsenic against trypanosomes were obtained soon after the identification of these micro-organisms. Lingaard in 1899 found that arsenious acid and other inorganic arsenic preparations cured a trypanosomal disease of horses, occurring in India, known as surra; while Bruce found that arsenious acid caused a temporary remission in tsetse-fly disease in Africa.

The distribution of drugs in the animal body had received attention from other workers besides Ehrlich, and the Meyer-Overton theory, propounded in 1899, established a reasonable scientific basis for this branch of chemotherapy (Meyer, 1899; Overton, 1901, 1902). Overton showed that chemical compounds may be divided into different groups according to the rapidity with which they diffuse into cell protoplasm, the rate of diffusion generally depending on the distribution coefficient of the compound between fat and water. Anæsthetics and narcotics usually diffuse rapidly into cells, and they possess relatively high distribution coefficients. Meyer showed that in the aliphatic narcotics, strength of narcotic action was approximately proportional to distribution coefficient.

Development of chemotherapy by Ehrlich

With this background, Ehrlich started on the experimental chemotherapy of trypanosome infections, largely because there was no known cure for sleeping sickness, which was becoming a major problem in the development of the continent of Africa. His first work in this field, published with Shiga in 1904, was the beginning of a systematic investigation of dyes as curative agents for trypanosomiasis in mice. Preliminary work on the benzopurpurin series of dyes, chosen apparently because of their long persistence in mice after injection, led finally to the discovery that trypan red was both curative and prophylactic for mice infected with the disease *Mal de Caderas* (*Trypanosoma equinum*) (Ehrlich, 1907). This was the first cure of an experimentally-produced disease by administration of a synthetic organic substance of known chemical composition. Trypan red had little practical use as it was relatively ineffective against other species of trypanosomes (e.g. *T. brucei*) in mice. However, the discovery led to the introduction by Mesnil and Nicolle (1906) of two other dyes of the same series, trypan blue and afridol violet, which were effective against *T. brucei* infections in mice and cattle. Here we have the first practical result of experimental chemotherapy; trypan blue was used to cure cattle of tsetse-fly disease, and was later shown by Nuttall and Hadwen (1909 *a* and *b*) to be effective in curing piroplasmosis in dogs and cattle.

Following work on triphenylmethane dyes by Wendelstadt and Fellmer (1906), Ehrlich found that malachite green had some curative action on experimental trypanosomiasis although it was fairly toxic, especially as a prophylactic. After examination of acridine dyes and the related oxazines and selenazines, a compound with considerable trypanocidal activity and low toxicity for mice was finally produced. This was diamino-methylacridinium chloride (trypaflavin, later known as acriflavine).

One important result of work with these dyes was the development *in vivo* of drug-resistant strains of trypanosomes.

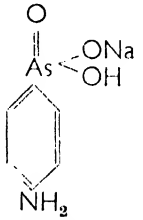
In Ehrlich's laboratory, Franke and Roehl found that feeding parafuchsin to mice infected with *Trypanosoma brucei* caused disappearance of trypanosomes from the peripheral blood, but, after a week or two, relapse occurred and the parasites reappeared. Administration of the drug caused them to disappear again. This process could be repeated at further relapses, but not indefinitely. After each successive administration, the time of banishment of trypanosomes from the blood became shorter, until finally the drug failed entirely to have an effect on the parasites. When trypanosomes from these mice were transferred to normal mice, they were found to be still unaffected by parafuchsin, and it was evident that the parasites themselves had acquired an increased resistance to the drug. This resistance was maintained consistently through numerous animal passages.

Later work by Ehrlich showed that such trypanosomes could acquire resistance to other known trypanocidal substances such as arsenical drugs. Strains of trypanosomes were developed which were resistant to certain compounds within one chemical class, but sensitive to other trypanocidal substances. This chemical specificity suggested that chemical processes were involved in the action of drugs on parasites, and led Ehrlich to put forward his "chemo-receptor" theory of drug action. This theory is, in essence, very similar to his other side-chain theories which we have already mentioned, and accounted for the action of a drug by its combination with a specific receptor in the parasite. Resistance to a particular drug was regarded as due to reduction of affinity of a receptor for one drug without change in combining powers for other drugs. This implies that parasites possess a whole series of chemo-receptors which are specifically different from one another, and suggests that a study of resistant strains should indicate the receptor on which a drug is acting.

Ehrlich's work on arsenical drugs

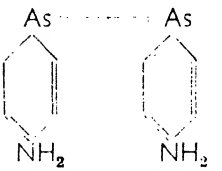
The field of organic arsenical drugs saw Ehrlich's most successful contribution to medicine and to chemotherapy. This work was based on his chemoreceptor theory in so far as

throughout its many stages, emphasis was continually laid on chemical combination of drugs both with the parasite and with the tissues of the host. In 1903 Ehrlich tested atoxyl against trypanosomes *in vitro*; it was without effect and he did not then test it on infected animals. Two years later, Thomas and Breinl (1905) found that atoxyl cured trypanosomiasis in mice, and trials by Koch in Africa showed that it cured the human form of trypanosomiasis known as sleeping sickness. Although atoxyl proved to be rather toxic to humans, occasionally causing optic nerve atrophy or other severe complications, it is important as the first cure to be discovered for sleeping sickness. The successful use of atoxyl *in vivo* revived Ehrlich's interest, and he started by re-investigating its structure, which was believed at this time to be an anilide of arsenic acid. In conjunction with Bertheim, Ehrlich showed that this conception was wrong, and that atoxyl is in fact the sodium salt of *p*-aminophenylarsonic acid (Ehrlich and Bertheim, 1907).



Atoxyl (p-*aminophenyl*-*arsonate*)

p-*Aminophenyl*-*arsenoxide*

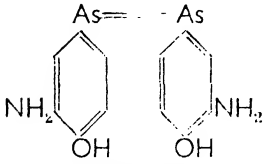


p-*Aminophenyl*-*arsenobenzene*

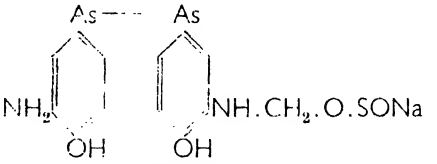
The significance of this constitution for atoxyl was at once recognised by Ehrlich, who saw the wide possibilities opened up through preparation of homologues by substitution in the amino group. However, before he had prepared many derivatives of atoxyl, he carried out an important investigation on the mode of action of the drug, searching for the reason for the failure of atoxyl to react on trypanosomes *in vitro*. In 1909 Ehrlich and Roehl showed that reduction of atoxyl by any ordinary reducing agent produced *p*-aminophenylarsenoxide. This compound, containing arsenic in the trivalent form, had a strong *in-vitro* trypanocidal action,

in contrast to the pentavalent atoxyl. Ehrlich (1909) postulated that the trypanocidal action of atoxyl *in vivo* is due to reduction of arsenic to the trivalent form by host cells. Therapeutic trials of *p*-aminophenylarsenoxide showed that it was highly toxic to both host and parasite. The arsenobenzene derivative, in which arsenic was also in the trivalent form, was found to be less toxic to animal cells, although almost as active as arsenoxide against trypanosomes.

Some evidence existed that arsenicals were also effective against spirochætes; other workers had found that atoxyl cured fowl spirillosis, also syphilis in apes, rabbits and even humans. Atoxyl had, however, no effect against relapsing fever, a prevalent, and hitherto intractable spirochætal disease. In view of the success of atoxyl against some spirochætal diseases, Ehrlich was convinced that other arsenic derivatives would be found to cure relapsing fever. Turning once more to the arsenobenzene derivatives, Ehrlich and Bertheim (1912) eventually prepared the hydrochloride of dihydroxydiaminoarsenobenzene, known as salvarsan. Some idea of the scope of their work may be derived from the serial number 606 given to this compound. Salvarsan proved to be successful in curing relapsing fever both in mice and humans, and was also found by Ehrlich and Hata (1911) to cure human syphilis and trypanosomiasis.



Salvarsan or Arsphenamine



Neosalvarsan or Neoarsphenamine

The more soluble derivative of salvarsan introduced by Ehrlich in 1912, known as neosalvarsan or neoarsphenamine, is a condensation product of salvarsan with sodium formaldehyde sulphoxide. After careful clinical trials, Ehrlich eventually allowed it to be put on the open market as an anti-syphilitic drug. It proved to be the greatest achievement of chemotherapy up to the discovery of the sulphonamides.

Salvarsan had a striking effect in curing frambœsia (yaws), another form of human spirochætal infection. By 1913 a hospital in Surinam in Africa, in which over 300 patients with frambœsia had been constantly under treatment, had to be closed for lack of patients as one injection of salvarsan sufficed to cure the disease. Unfortunately, syphilis is not so easily cured, but, provided the disease is caught in its early stages and vigorous treatment is given, a cure can be fairly certainly effected by neoarsphenamine together with bismuth.

Ehrlich's theories of drug action

Throughout his work on chemotherapy, Ehrlich was guided by his chemo-receptor theory of drug action, together with other principles or beliefs which helped him in his selection of drugs for trial (summarised by Ehrlich, 1913). The first principle was " *Corpora non agunt nisi fixata* " ; in other words, parasites were only attacked by drugs which they fixed by means of their chemo-receptors. Apparent exceptions to this principle existed, since in many cases no direct action of a drug on the parasite *in vitro* could be observed. Ehrlich explained this by supposing that the amount of drug fixed by parasites, although insufficient to kill immediately, was sufficient to prevent multiplication of parasite in the host, thus permitting phagocytic action to eliminate the infection. He showed that although spirochætes treated with salvarsan *in vitro* remained fully motile after washing free from the drug, they would not infect an animal into which they were injected. This point of view was ignored for many years, but is to-day considered to represent the most probable mode of action of most chemotherapeutic drugs which are bacteriostatic rather than bactericidal.

We should emphasise here that although Ehrlich employed a somewhat pictorial representation of reaction of drug with its receptors, his own conception of the interaction was essentially a chemical one, in which the forces involved were those of ordinary chemical reaction.

The fixing of drug by parasite Ehrlich termed a " parasitotropic " effect. Most drugs were also toxic to the host and

were supposed to be fixed on the chemo-receptors of the host cells; this tendency was termed "organotropic." Ehrlich emphasised that drugs suitable for chemotherapeutic use were those in which the ratio between organotropic and parasitotropic effect was sufficiently low to avoid poisoning of the host. The ratio was ascertained experimentally by comparison between the "*dosis toxica*" and the "*dosis tolerata.*" Here we see the genesis of what is now known as the chemotherapeutic index; the ratio of maximum tolerated dose to minimal effective dose. This ratio is an important factor in determining the clinical success or failure of any new drug.

In arsenical drugs Ehrlich distinguished two types of groupings—the trivalent arsenic group, responsible for the toxic effect, and a "fixative" group which fixed the drug to the parasite and so allowed the toxic group to act. The fixative group was also supposed to determine the organotropic character of the drug. In Ehrlich's opinion the aim of chemotherapy should be to achieve "*Therapia magna sterilans.*" or complete recovery with one massive dose of drug. This is a very desirable aim, which has not yet been achieved by our modern chemotherapeutic drugs. Whether it will ever be attained is a matter for the future to decide.

Bacterial chemotherapy

Ehrlich confined most of his chemotherapeutic studies to diseases of protozoal or spirochætal origin, and did little work on the chemotherapeutic treatment of bacterial infections, but experiments which he did carry out were of considerable theoretical importance. In collaboration with Bechhold, he investigated the germicidal powers of substituted phenols (Bechhold and Ehrlich, 1906; Bechhold, 1909). Compounds were obtained which far exceeded all previously known phenols in their bactericidal action *in vitro* in nutritive broth, but therapeutic experiments on infected animals proved unsuccessful. This failure was shown to be due to the fact that these disinfectants combined with serum proteins to such an extent that their germicidal power was depressed.

The first cure of a bacterial infection, as distinct from a

spirochætal, was reported by Morgenroth and Levy (1911). The quinine derivative, ethylhydrocuprein or "optochin," which had a powerful and specific inhibitory action on pneumococci *in vitro*, was also found to cure mice infected with pneumococci. Unfortunately, extension of the work to human cases did not show similar cures and the substance was found to be very toxic. Later work by Morgenroth showed that homologues of optochin were highly active against other bacteria *in vitro*; for example, *iso*amylhydrocuprein (eucupin) was strongly germicidal for diphtheria bacilli, while the *iso*octyl derivative (vucin) was active against streptococci. These compounds were not active against general infections in animals, but were found to have some local antiseptic action when introduced into the site of infection. These observations of Morgenroth were the starting point for a considerable amount of work on quinine derivatives, which has not, however, resulted directly in the development of any successful chemotherapeutic agent for bacterial diseases.

During the early part of the twentieth century investigations were continued on the bactericidal action of dyes. Churchman was concerned with the selective action of gentian violet on 130 bacterial species, and divided the organisms into violet-positive and violet-negative according to whether or not their growth was inhibited by the dye (Churchman, 1912, 1913; Churchman and Michael, 1912). Simon and Wood (1914) extended their observations to a great variety of dyes and found that only basic dyes had an inhibitory action, no effect being obtained with acidic dyes. The development of strains of organisms resistant to various dyes was described by these authors and the existence of natural dye-fast strains was also noted. This was not the first case of bacterial resistance to be described, as resistance to drugs had been observed in the nineteenth century, and Morgenroth a few years previously had produced strains of pneumococcus resistant to optochin. However, the publications of Simon and Wood are interesting as they contain a theory of drug action and resistance which differs somewhat from Ehrlich's theory, and

bears a marked resemblance to some of our modern theories. They ascribed, as Ehrlich did, the inhibitory action of dyes to the existence of receptors in the micro-organism to which the dye was anchored—the receptors probably being acidic groups which reacted with the basic dyes. The receptors were considered to be " nutriceptors " responsible for carrying on the metabolism of the cell, and since they were blocked by combination with dye, the cell died, not necessarily because it had been poisoned, but because a sufficient number of its nutriceptors had been thrown out of action to bring about its starvation or inability to multiply. Adaptation to grow in the presence of inhibitory amounts of dyes was ascribed to the existence of other receptors by which the organism could carry out its nutrition and reproduction, and to the possibility of the organism " producing such receptors while the others were occupied by the dye." No suggestion was made as to the nature of these receptors, but the remark is made that " since intracellular metabolism is intimately connected with the action of the enzymes, the question has naturally suggested itself whether the deleterious action of the dyes may not in part be referable to interference with the activity of these components."

Browning and his co-workers showed that the acridine dye, acriflavine, had a strong antibacterial action which was augmented, rather than decreased, by the presence of serum ; general toxicity was relatively low (Browning and Gilmour, 1913 ; Browning, Gulbransen and Thornton, 1917 ; Browning and Gulbransen, 1917). Here again Ehrlich's influence is evident ; he had introduced the same compound, under the name trypaflavine, as a trypanocidal substance.

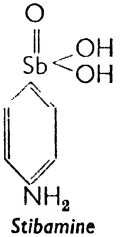
Another acridine dye, 2-ethoxy-6 : 9-diaminoacridine, was found by Morgenroth, Schnitzer and Rosenberg (1921) to be even less toxic than acriflavine and to possess a high bacteriostatic action. Its hydrochloride, known as rivanol, could be injected safely into humans. Morgenroth managed to cure mice of a streptococcal infection by rivanol, but only by injection immediately after infection and close to the site of application of the organism. In other words, the action was

only a local one. Both acriflavine and rivanol have since proved of value for local applications because of their low toxicity for tissues. The chemotherapy of systemic bacterial infections was, however, slow in developing, and no real advances were made in this field until the introduction of the sulphonamides in 1935.

Protozoal and spirochætal chemotherapy

Following Ehrlich's discoveries, further slow progress was made in the chemotherapy of protozoal and spirochætal infections. The trypanocidal effect of tartar emetic had been demonstrated in 1908 (Mesnil and Brimont, 1908; Plimmer and Bateman, 1908); it has proved to be of some value in the treatment of trypanocidal diseases in domestic animals and even in humans. In 1912 Gaspar Vianna opened up the field of chemotherapy of leishmanial infections by showing that dermal leishmaniasis in Brazil could be cured by tartar emetic. This was confirmed for other leishmanial diseases in different parts of the world, and specific treatment of these diseases with antimony was soon widespread. Kala-azar is probably the most serious and widespread of leishmanial infections; before the introduction of antimony therapy, the death-rate in India among sufferers from this disease was almost 90 per cent.

Both sodium and potassium antimonial tartrates were found to give rise to toxic effects, and these were soon discarded in favour of the less toxic pentavalent organic preparations which are derivatives of phenylstibonic acid.



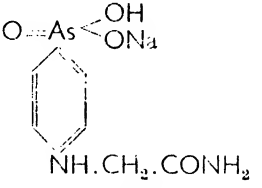
Stibamine

The first of these compounds to be tested was *p*-aminophenylstibonate (stibamine), the antimony analogue of atoxyl

(Uhlenhuth, Mulzer and Hügel, 1913); it proved to be too unstable in solution and too uncertain in action to be used therapeutically. Its acetyl derivative, stibenyl, was successfully employed by Caronia in 1916 for the treatment of kala-azar in Italy, but it has been found to be ineffective in India.

After this, slow but steady advance was made in the chemotherapy of diseases of spirochætal and protozoal origin. Bismuth was introduced for the treatment of syphilis by Sazerac and Levaditi (1921). They showed the therapeutic activity of sodium potassium tartrobismuthate in rabbit syphilis and in some human cases. Actually, the use of bismuth in syphilis was first suggested by Balzar (1889), but it does not appear to have been tried out; it was found to be curative in fowl spirochætosis by Robert and Sauton (1916) but its value again passed unnoticed until 1921. Since then, more than 200 new bismuth compounds have been produced, often with little or no preliminary study to determine whether they were an improvement on already known compounds. The action of organo-bismuth compounds appears to be due to the liberation of metallic bismuth, and the value of different preparations seems to depend on their power of penetrating the parasite cell.

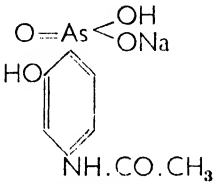
Jacobs and Heidelberger (1919), in attempting to find a satisfactory trypanocidal compound, prepared a large number of organic arsenical compounds, and from these selected sodium N-phenylglycineamide-*p*-arsonate, now known as



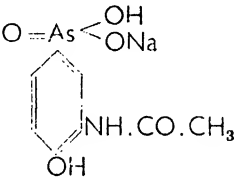
Tryparsamide

tryparsamide. This compound proved to be the first reasonably satisfactory drug for the treatment of human sleeping sickness, but it was not without toxic effects.

Fourneau and his colleagues made a study of the arsonic acids, particularly those containing the hydroxyl and amino radicals which are present in salvarsan. They prepared all the nine isomers of salvarsan and also the acetylated derivatives. The result of this was the introduction of orsanine and stovarsol, two isomers of sodium hydroxy-acetylamino-phenylarsonate (Fourneau, Navarro-Martin, Tréfouël and Tréfouël, 1923). Orsanine, *p*-acetylamino-*o*-hydroxyphenyl-



Orsanine

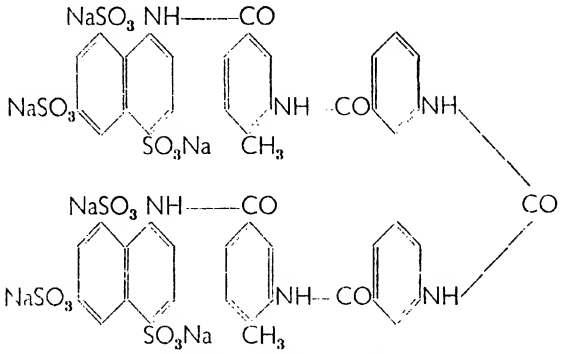


Stovarsol

arsonate, has a strong trypanocidal action, and until the discovery of amidines, has shared with tryparsamide the position of being the only satisfactory drug for the treatment of sleeping sickness in its later phases. Stovarsol, *p*-hydroxy-*m*-acetylaminophenylarsonate, has no action on trypanosomes, but has fairly strong anti-spirochætal and amœbicidal actions, and has been occasionally used where injection is impracticable, as it can be administered orally. Fourneau's work is also of considerable theoretical interest, as it illustrates well the high specificity of drug action.

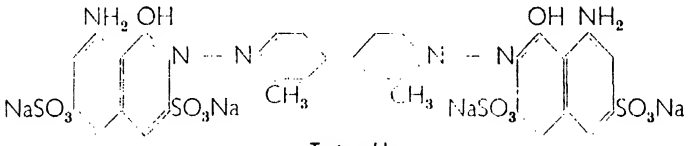
Hitherto, synthetic chemotherapy had been chiefly concerned with organo-metallic trypanocidal and anti-spirochætal drugs. The introduction in 1920 of Bayer 205 (Germanin or Moranyl) marked an important departure from this field. The constitution of the drug, synthesised by the German firm of Bayer, was originally withheld; but a fine piece of work by Fourneau (1924), involving the synthesis of many ureas of the acylnaphthylamine-sulphonic acid type, led to a compound (Fourneau 309) with the same therapeutic properties as Bayer 205. Fourneau found that the chemical specificity of his compound was most marked; the slightest change in structure resulted in a diminution of trypanocidal activity.

He therefore concluded that his substance had the same structure as Bayer 205.

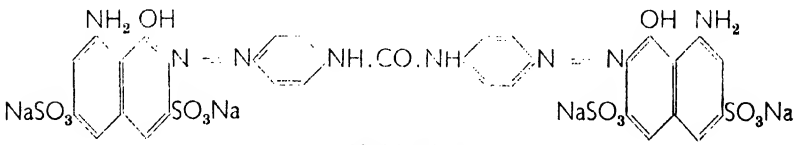


Bayer 205 or Fourneau 309

The drug is said to have been produced by Bayer in a search for a colourless substance related to the trypanocidal dyes, trypan blue and afridol violet, which were introduced in 1906 by Mesnil and Nicolle. Trypan blue was used for the treatment of trypanosomiasis in cattle, but, since bright blue meat was unsaleable, the Bayer company attempted to find a colourless trypanocide. Afridol violet already contained



Trypan blue



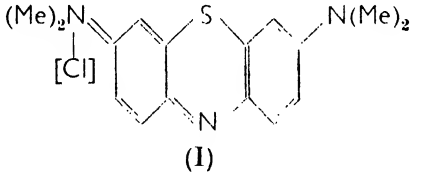
Afridol violet

one urea linkage; the further use of amide linkages instead of azo groups provided a colourless substance.

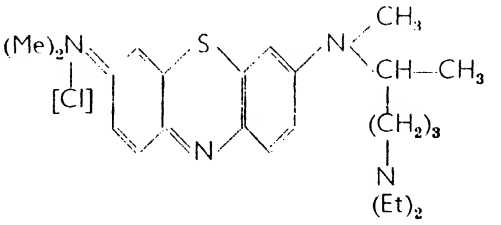
Bayer 205 has proved to be an extraordinarily useful trypanocidal agent; its chemotherapeutic index for mice is about 300, the highest for any compound known at that time.

It is interesting to note that Ehrlich (1913) considered a therapeutic index of 10 or even 5 was as high as could be expected. One disadvantage of the drug is that it is unable to cure sleeping sickness in the advanced stages when trypanosomes have attacked the brain, since it cannot pass into the cerebrospinal fluid. However, provided that it is given in the first few weeks after the beginning of the illness, there is a reasonable chance of cure. Later stages of the disease must still be treated with either tryparsamide or orsanine, both of which penetrate to the cerebrospinal fluid. A useful feature of Bayer 205 is its persistence in action, probably due to its slow rate of excretion. When administered prophylactically, a single dose may confer immunity to sleeping sickness for several months.

Other successful investigations originating from the work of Ehrlich were in the antimalarial field. It will be remembered that Ehrlich in 1891 found that methylene blue (I) had some curative effect on malaria. The Bayer research chemists accordingly tried the effect of replacing the methyl groups attached to the aromatic amino groups of methylene blue



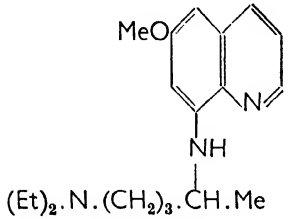
(I)



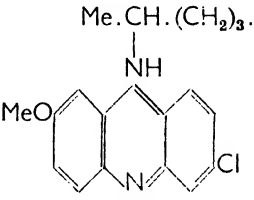
(II)

with other alkyl groups. Next they tried replacing the methyl groups with basic alkyl groups, producing compound (II) (see formulæ) with the favourable chemotherapeutic index of 8.

The work on methylene blue did not produce a compound of any practical value, but it indicated some relationship between antimalarial effect and chemical structure. The experience gained was applied to other ring systems, with the result that plasmoquine, a derivative of 6-methoxy-8-aminoquinoline with a therapeutic index of 30, was produced in 1924 (Schulemann, Schönhöffer and Wingler, 1932). Success in the quinoline



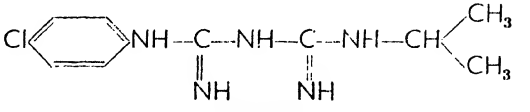
Plasmoquine



Atebrin

series naturally led to an examination of similar substitutions in other heterocyclic nuclei, and led eventually to the acridine derivative known as atebrin or mepacrine (Mauss and Mietzsch, 1933). This substance is very effective against human malaria, both prophylactically and curatively, and has a low toxicity. It proved to be superior to quinine during the war of 1939-45, when it was shown by field trials to be a prophylactic against benign and malignant tertiary malarias and a cure for the malignant tertiary form.

Atebrin has now been supplemented by paludrine, an antimalarial drug synthesised by chemists of Imperial Chemical Industries of Great Britain in 1944 (Curd and Rose, 1946).



Paludrine

Paludrine is a biguanidine compound, the result of a search for antimalarial drugs among pyrimidine derivatives. The pyrimidines were found to have antimalarial properties if they possessed configurations which permitted certain tautomeric changes. Working on the theory that this tautomeric capacity conferred antimalarial activity, the pyrimidine ring was found

to be unnecessary, and biguanidine derivatives with high activity were produced.

Paludrine is proving to be a most potent and non-toxic antimalarial drug. It is active against all three forms of malaria, and possesses the property, absent from other antimalarial drugs, of preventing the development of parasites in the pre-erythrocyte stage following primary infection (Fairley, 1946).

Pharmacological basis of chemotherapy

The successful development of chemotherapy, as we have described it, appears at first sight to be a purely empirical process based upon patient trial of innumerable analogues of a compound of known activity. This is not strictly true; since 1920 there has gradually developed a pharmacological basis for chemotherapy concerned with the action, distribution and excretion of chemotherapeutic drugs in the body. Much of the pioneer work on this subject was carried out by Voegtlin between 1921 and 1930 (Voegtlin, 1925). He emphasised the importance of this side of chemotherapy since it determined to a great extent whether or not a drug which was active *in vitro* would be similarly active *in vivo*. He showed that parasiticidal action *in vivo* is dependent not only on the toxicity of drug for the parasite, but also on the rate of excretion of drug and its power of penetrating tissues. He attempted to explain the differential action of drugs in poisoning parasites rather than the host, a fact simply accepted by Ehrlich. Voegtlin suggested that either the capacity of host cells to convert the toxic form of a drug into a non-toxic form was greater than that of parasites, or that parasites had a greater permeability for the drug than the host cells.

Voegtlin provided the first practical demonstration of Ehrlich's theory that the pentavalent form of an arsenical drug is converted by the host into the active trivalent oxide form; he also established the point that arsenobenzene derivatives are not active as such but are transformed in the host to the active arsenoxide form. This point seems to

have escaped Ehrlich in his development of salvarsan (Voegtlin, Dyer and Leonard, 1923). When salvarsan or other arsenobenzene derivative was injected into a rat infected with trypanosomes there was a latent period of several hours before destruction of the parasite, but the trivalent oxide form of salvarsan had an immediate trypanocidal action. The toxic effect of arsenoxides was shown to be due to their action on sulphydryl groups in the host tissues, since glutathione, a sulphydryl-containing peptide, protected an animal when injected immediately after a lethal dose of arsenoxide. Glutathione and various other organic sulphydryl compounds were also found to protect trypanosomes both *in vitro* and *in vivo* from the lethal effect of arsenoxides. A direct practical result of this work of Voegtlin was the introduction, as a therapeutic drug, of the arsenoxide derivative of salvarsan, under the name mapharsen (*m* - amino - *p* - hydroxyphenylarsenoxide). As with other arsenoxides, toxicity is high, a fact which caused Ehrlich to discard the compound as a practical drug; nevertheless, because of its potent action on parasites, mapharsen possesses a favourable therapeutic index.

Voegtlin's ideas do not appear to have taken root, for much speculation continued for some years on the nature of chemotherapeutic action. Did drugs act directly on the parasite, or did they act indirectly through the host, either by stimulating the defence mechanism, or by giving rise to the production by the host of a parasiticidal substance? The main reason for the persisting doubt appears to have been the absence of correlation between *in-vivo* and *in-vitro* action of drugs. Some drugs appeared to have no direct killing action *in vitro*, but undoubtedly countered the organisms *in vivo*. Equally puzzling was the *in-vitro* action of emetine and cephaeline, the chief alkaloids of ipecacuanha, known to be effective cures for amœbic dysentery. Dale and Dobell (1917) found that these alkaloids were less toxic to isolated dysentery amœbæ than other alkaloids which were not curative for the disease. This lack of correlation between *in-vitro* and *in-vivo* observations was a direct result of inability to culture or

even maintain the organisms in question outside the animal body. The time of survival of trypanosomes or dysentery amœbæ in the media used at that time was a matter of a few hours, and it is obvious that no extended observations were possible on parasitic reactions to drugs. Similarly, the effects of drugs on growth and reproduction could not be determined, and the theory originally propounded by Ehrlich, suggesting that drugs acted by preventing multiplication, was discarded for want of positive evidence (review by Dale, 1923).

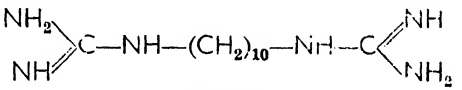
The introduction of a culture medium for dysentery amœbæ by Boeck and Drbohlav (1925) enabled Dobell and Laidlaw (1926) to show that emetine and cephaeline were far more poisonous than other alkaloids under suitable cultural conditions, and that very dilute solutions, although not immediately toxic, were lethal to the organisms. The concentration required to kill immediately bore no relation to the minimal concentration which was lethal if maintained for a period of days. These observations accounted for the anomalous results of Dale and Dobell. Later Laidlaw, Dobell and Bishop (1928) were able to cultivate the organisms in a purely liquid medium, in which a concentration of emetine of 1 in 5,000,000 was sufficient to inhibit growth.

The problem of *in-vitro* cultivation of trypanosomes has not yet been completely solved, but Yorke, Adams and Murgatroyd (1929) developed a medium which would maintain trypanosomes alive and motile for twenty-four hours. This opened up the field of trypanosome therapeutics considerably and has led to a better understanding of drug action in these organisms. At the same time, these workers found that trypanosomes consume enormous amounts of glucose *in vitro* —400 million trypanosomes (representing about 27 mg. of material) causing between 2·0 and 2·5 mg. of sugar to disappear in 1 hour at 37° C. This fact, fully confirmed by later work, led directly to the important discovery of the amidine group of drugs. In discussion of the *in-vitro* cultivation of trypanosomes it must be emphasised that the difficulty lies, not in the subculture of viable protozoa, but in the

maintenance of pathogenic strains in the form in which they exist in the animal host (*cf.* Weinman, 1946 ; Brand, Johnson and Rees, 1946).

Amidines

Jancsó and Jancsó (1935*b*) concluded that the trypanocidal action of Bayer 205 was due to its interference with the carbohydrate metabolism of trypanosomes, as the drug produced changes in trypanosomes similar to those resulting from removal of sugar from the medium in which the parasites were suspended. In view of the high carbohydrate metabolism of trypanosomes, they decided to examine the reaction of parasites to guanidine derivatives, which were known to lower blood sugar levels in animals (Frank, Northmann and Wagner, 1926). Several of these derivatives (especially



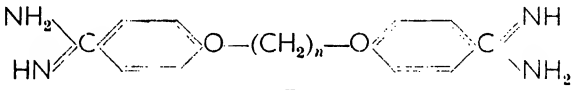
Synthalin

synthalin) were found to exert a therapeutic action on mice infected with *Trypanosoma brucei*. The Jancsós concluded that the therapeutic effect was an indirect one, due to continuous hypoglycæmia depriving the trypanosomes of the necessary glucose for their development. Schern and Artagaveytia-Allende (1936) showed independently the therapeutic action of synthalin in rats infected with trypanosomes.

Lourie and Yorke (1937) considered it improbable that a degree of hypoglycæmia compatible with life of the host would be sufficient to affect the parasite adversely, so they examined the *in-vitro* effect of synthalin on trypanosomes. This was found to be extremely powerful, a concentration of 1 in 200 millions having a pronounced trypanocidal action. However, insulin had no effect on trypanosomes either *in vitro* or *in vivo*, while synthalin produced little hypoglycæmia in the normal animal unless given in doses so large as to cause liver damage. They therefore concluded that synthalin exerted a direct toxic effect on trypanosomes.

To test this theory, a large number of guanidines, isothioureas, amidines and amines, with alkyl and alkylene chains, were prepared and examined for trypanocidal activity (King, Lourie and Yorke, 1938). Certain of the diamidines showed a powerful trypanocidal action both *in vitro* and *in vivo*; the most active compound, undecane-diamidine, producing almost 100 per cent. cures in mice and rabbits infected with *T. rhodesiense*.

The firm of May and Baker later produced a series of aromatic derivatives containing the amidine group. Some of these have proved to be valuable chemotherapeutic drugs (Ashley, Barber, Ewins, Newbery and Self, 1942). The 4 : 4-diamidinodiphenoxyalkanes (I where $n = 3$ or 5) showed low toxicities, 4 : 4-diamidinodiphenoxypentane having a therapeutic index of 15. 4 : 4-Diamidinostilbene (II) was even less toxic with a therapeutic index of 30.



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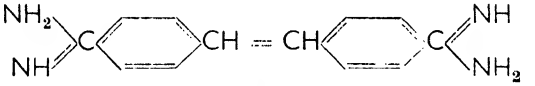
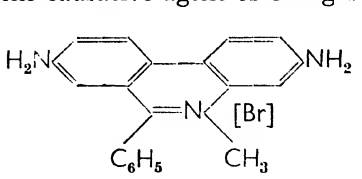


II

The amidines are fairly unspecific in their action on protozoa, and have been shown to have a therapeutic action in trypanosomiasis, leishmanial infections such as kala-azar, piroplasmosis such as *Babesia canis*, and malaria infections of birds and monkeys. 4 : 4-Diamidinostilbene acts effectively against the early stages of sleeping sickness, but, like Bayer 205, is unable to penetrate into the cerebrospinal fluid, and is thus of little value in the later stages. It is very effective against kala-azar, but, although it has an antimalarial action, it is of little use in treating human malaria. The amidines have no action against spirochætes.

Treatment of trypanosome infections in cattle has been extended by the discovery that certain phenanthridines are curative against infections caused by *Trypanosoma congolense*

(review, Walls, 1947). The most useful compound, dimidium bromide, has the structure indicated below (I). Another closely related compound (II) has some action against *T. cruzi*, the causative agent of Chargas' disease.



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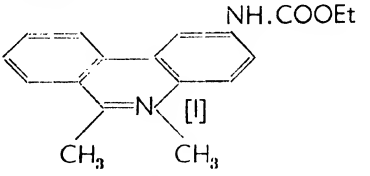
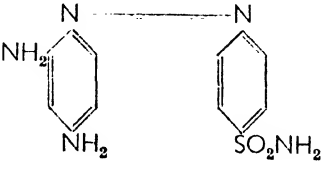


II

Development of Sulphonamides

In the early 'thirties, as far as the treatment of bacterial infections was concerned, little of value had come out of chemotherapy. In 1930 the view was being expressed that bacteria were not susceptible to destruction by drugs in the host, possibly because their metabolism was so similar to that of host cells that anything capable of toxic action against bacteria was sure to be equally toxic to the host tissues. Serological treatment was considered to be the only method of combating bacterial infections and a considerable amount of research was done in this field. The introduction of prontosil in 1935 changed the situation, and it was soon realised that a new era in bacterial chemotherapy had opened.

The use of a drug called "streptozon" on a ten-month-old infant dying of staphylococcal septicæmia was reported by Foerster (1933) to have produced a dramatic cure. Streptozon was produced by the firm of I.G. and was the compound later known as prontosil (4'-sulphonamido-2 : 4-diaminoazo-



benzene). Patent cover for this compound had been obtained in 1932. Domagk of I.G. at that time observed that, when given by mouth, prontosil prevented the evolution of otherwise

fatal hæmolytic streptococcal infections in mice, cured chronic streptococcal infections in mice, and favourably influenced staphylococcal infections in rabbits, but had no effect on pneumococcal or other experimental infections and was without action *in vitro* on bacteria. This work was not published till 1935 (Domagk), but various reports of clinical trials of the substance in 1934 showed that it cured erysipelas and streptococcal empyema. Clinical trials reported in 1935 established that prontosil is highly effective against most hæmolytic streptococcal infections, particularly puerperal fever. A chemotherapeutic agent for bacterial infections had been found.

The development of prontosil can be traced back to work on azo dyes. In 1909, Hörlein of I.G., working on textile dyes, synthesised azo dyes with sulphonamide and substituted sulphonamide groups. These dyes were characterised by the stable complexes which they formed with wool proteins. Later, Eisenberg (1913) found that the dye chrysoidin (2 : 4-diaminoazobenzene) was bactericidal *in vitro* but had little effect in the living animal. In an attempt to increase the bactericidal properties of quinine derivatives, dyes based on hydrocupreine were prepared by Heidelberger and Jacobs (1919); one of these was *p*-aminobenzenesulphonamido hydrocupreine. The authors commented on the high bactericidal potency of these compounds but published no further experimental work on the subject. Work by Mietzsch and Klarer of I.G. on azo compounds led to the development of compounds of greatly increased bactericidal power when compared with known compounds, but they had no effect *in vivo*. However, Domagk observed that azo compounds containing sulphonamide had slight activity in combating streptococcal sepsis in mice. This observation directed further work into the channels eventually leading to prontosil.

After the publication by I.G. of their results in 1935, Levaditi and Vaisman (1935) in France confirmed the results on mice with a preparation of prontosil prepared for them by Girard and called "rubiazol." Soon came the suggestion by Tréfouël, Tréfouël, Nitti and Bovet (1935) that prontosil was broken in the host tissues at the azo linkage, yielding

p-aminobenzenesulphonamide, which was the therapeutically active principle. The basis for this suggestion was the fact that diazotised benzenesulphonamide derivatives quite different from prontosil still possessed anti-streptococcal properties, while derivatives in which *p*-aminobenzenesulphonamide was replaced by other groups were inactive.

No great attention was paid in the medical literature to these important advances until the appearance of two English papers in 1936. Colebrook and Kenny (1936) showed that although prontosil was inactive in the test tube, it did produce an increase in the bacteriostatic power of the blood of patients. They also established the value of prontosil therapy in puerperal sepsis. Buttle, Gray and Stephenson (1936) confirmed the French findings that *p*-aminobenzenesulphonamide (sulphanilamide) had a curative effect on streptococcal infections in mice, and showed that it was also of value against meningococcal infections. The French workers later showed that sulphanilamide exerted a bacteriostatic effect on susceptible organisms *in vitro*, without being immediately lethal. The *o*- and *m*-aminobenzenesulphonamides and *p*-acetyl sulphanilamide were without sulphanilamide effect (Nitti, Bovet and Depierre, 1937). Finally, Fuller (1937) confirmed the suggestion of Tréfouël and co-workers that prontosil was split in the host to *p*-aminobenzenesulphonamide.

Many other sulphonamide derivatives have been prepared and found to be more active than sulphanilamide and to combat a wider range of infections. The first really effective one was sulphapyridine or "M and B 693," prepared by May and Baker Ltd., and tested by Whitby (1938). It was shown to be particularly effective against pneumococcal infections. Research in the sulphonamide field still continues to-day, and is opening up ever-wider aspects in chemotherapy as more active and less toxic drugs are found, with a greater range of action over different bacterial types.

Antibiotics

The sulphonamides were the first class of drug to be used clinically against bacterial infections. Lately we have seen

the development of a new type of chemotherapeutic agent in the *antibiotics*. These are soluble antibacterial substances produced by micro-organisms during growth on suitable media.

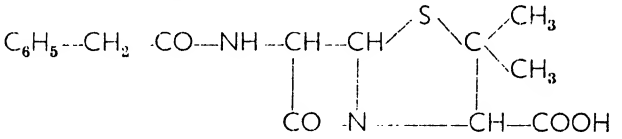
Since the beginning of bacteriology, inhibition of growth of one bacterial species by the presence of another micro-organism was an established fact, and its possible application to the field of therapeutics was realised. Pasteur and Joubert (1877) noted the antagonistic effect of aerobic bacteria on the growth of *Bacillus anthracis*, and even found that death of animals from anthrax could sometimes be prevented by including some of these aerobic bacteria in the infective dose of *B. anthracis*. Pasteur ascribed the effect to the consumption of oxygen by the aerobic organisms, but Babès (1885) interpreted experiments on growth inhibition of one organism by another as due to a chemical substance produced by the antagonistic organism. Emmerich and Low (1899) prepared an extract of *Pseudomonas pyocyanea* to which they gave the name pyocyanase. Highly diluted pyocyanase was found to have a destructive effect against pathogenic cocci, and against diphtheria, cholera, typhoid and plague organisms. These authors suggested that pyocyanase might be of use clinically, but it proved too toxic except for local application. Attempts to use pyocyanase clinically were continued for about twenty years but it gradually fell into disrepute as a remedy, possibly owing to the loss or degeneration of the original antibiotic-producing strain. There is no doubt that *Pseudomonas pyocyanea* can produce substances which are highly bacteriostatic, though it remains to be seen whether they have any clinical application (Hays *et al.*, 1945). Bacteriolytic substances have also been obtained from bacteria and tested therapeutically. Nicolle (1907) isolated from *Bacillus subtilis* a thermostable substance with lytic action against many pathogenic organisms. A considerable amount of work was subsequently carried out on these bacterial lysins in the hope that they could be used therapeutically, but they have proved toxic to animals. However, Gratia and Dath (1924, 1925, 1926) extracted a lytic agent from streptothrix mould which

had been grown on dead *Staph. aureus*, and this substance was employed, together with a bacteriophage, in the successful treatment of *Staph. aureus* carbuncles.

Many fungi have been found to produce substances with antibacterial action. Gosio (1896) produced from a *Penicillium* a crystalline substance which inhibited the growth of anthrax bacilli. He was unable to carry out tests on animals owing to lack of material, but the substance has since been re-isolated under the name mycophenolic acid and found to be useless as a chemotherapeutic agent although active *in vitro* (Florey, Gilliver, Jennings and Sanders, 1946). Filtrates from *Aspergillus fumigatus* were found by Vaudremer (1913) to cause attenuation of *Mycobacterium tuberculosis*, and were used to treat human tuberculosis, apparently with some success. Recent work has shown that this mould produces four antibiotic substances, one of which, helvolic acid, has some action against the tubercle bacillus *in vitro* (Chain, Florey, Jennings and Williams, 1943; Jennings, 1945).

In 1929 Fleming observed that on certain agar plates in which *Staph. aureus* was contaminated by a mould, the staphylococcus colonies were transparent and undergoing lysis. He identified the mould as a *Penicillium* and found that broth in which it had been grown had bactericidal and bacteriolytic properties against pyogenic cocci and the diphtheria group of bacilli, but was inactive against Gram-negative organisms such as *Hæmophilus influenzæ*, *Escherichia coli* or *Eberthella typhosa*. The name penicillin was given to active filtrates of the broth. Fleming showed that it was almost completely non-toxic to animals, to humans, and to leucocytes, and therefore suggested that it would be a useful antiseptic for infected wounds. Subsequent attempts by Clutterbuck, Lovell and Raistrick (1932) to concentrate the active principle proved unsuccessful because of its instability and low concentration in culture filtrates. These authors showed, however, that the mould could be grown on a synthetic liquid medium. Eventually there was isolated from the culture medium of *P. notatum* a water-soluble powder with remarkable antibacterial activity (Chain, Florey, Gardner,

Heatley, Jennings, Orr-Ewing and Sanders, 1940; Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941). The material was effective *in vivo*, subcutaneous injections producing 100 per cent. cures of mice infected with *Strep. hæmolyticus*, *Staph. aureus* or *Clostridium septicum*. Florey and his school have continued to carry out chemical, bacteriological and pharmacological work on penicillin, with the result that it is now produced on a vast scale for the treatment of many bacterial infections. The chemistry of penicillin has been worked out in many laboratories; several forms of the substance are known, one of which, benzyl penicillin (penicillin G), has the following constitution (see review by du Vigneaud *et al.*, 1946).



Benzyl Penicillin

Penicillin is bacteriostatic to most Gram-positive organisms, and also to spirochætes; it has little or no action against Gram-negative organisms. It is highly active, being bacteriostatic in dilutions greater than 1 in 10^8, and is completely non-toxic. Its activity is not affected by tissue proteins or breakdown products, or pus, and is relatively unaffected by the number of bacteria present. In these respects it is an ideal chemotherapeutic agent. It is, however, very rapidly excreted in the urine, and cannot be taken by mouth as it is destroyed by acid. It must therefore be administered in large and frequent doses by injection or intravenous drip. Other disadvantages of penicillin are its extreme lability, low yields and high cost of production. These disadvantages are all outweighed by its low toxicity and wide range of application, with the result that penicillin has widened the field of bacterial chemotherapy to an extent which would have been unbelievable fifteen years ago. The synthesis of penicillin has proved to be very difficult, with yields of the order of 0·2 per cent. However, when full investigations have been

carried out on the groups essential for activity, it is to be hoped that derivatives will be synthesised with all the advantages of penicillin, but with few of its disadvantages.

The isolation by Dubos and Hotchkiss (1941) of the two antibacterial substances gramicidin and tyrocidine from *Bacillus brevis* was the result of rational attempts to produce antibacterial substances. Dubos started by attempting to find a microbial enzyme able to attack the Gram-positive substance in pyogenic cocci. This was done by serial subculture of soil organisms in a medium containing large amounts of living organisms. An organism (*B. brevis*) capable of using coccal protein as a source of food was thus selected (Dubos, 1939). Bacteriostatic substances were found in the growing culture, and were shown to be more or less stable alcohol-soluble toxins which the organisms released on autolysis (Dubos and Cattaneo, 1939). From the alcohol extract, the antibacterial substances gramicidin and tyrocidine were isolated by crystallisation. These are both polypeptides of molecular weight 3000 or less, containing relatively large amounts of *d*-amino acids (gramicidin contains 50 per cent. of *d*-amino acids) (Hotchkiss, 1944). Tyrocidine has many basic groups and shows antibacterial properties similar to those of the cationic detergents, *i.e.* combining with or precipitating protein generally; it is therefore of little use therapeutically. Gramicidin has no excess of free acidic or basic groups, and is somewhat less toxic; also, it is bacteriostatic rather than directly lethal. It is only of use clinically when applied at the site of infection as it is too toxic for general use. Recently, Russian workers have reported the isolation of another antibiotic polypeptide, gramicidin-S (Belozersky and Passhina, 1944). It is believed to be a cyclic deca-peptide, with the following arrangement of amino acids: *l*-valine, *l*-ornithine, *l*-leucine, *d*-phenylalanine, *l*-proline (Consden, Gordon, Martin and Synge, 1947).

An antibiotic substance known as streptomycin has been isolated from an *Actinomycete* by Schatz, Bugie and Waksman (1944). It is highly active against some organisms not attacked by penicillin, such as *Mycobacterium tuberculosis* and the Gram-negative *Escherichia coli* and *Pseudomonas*

pyocyanea. Its therapeutic value against tuberculosis is at present under investigation.

The subject of antibiotics is very much in its infancy. Isolations and trials of new substances obtained from micro-organisms are continually being reported. The chemistry of antibiotics is a large field in itself, and should yield much valuable material for future building stones of the science of chemotherapy (review, Benedict and Langlykke, 1947).

CHAPTER II

CELL METABOLISM

THE early successes achieved by chemotherapy tended to obscure the empirical nature of the researches leading to these results. The chief reason for this empiricism was that, until comparatively recently, research was concerned mainly with the action of drugs when injected into infected animals. This method, while it gave satisfactory practical results, involved such a host of unknown variables that no real advance in knowledge of the mode of action of drugs was possible.

For chemotherapy to grow as a science, attention must be devoted to the fundamental aspects of drug action. Drug distribution in the host must be understood ; the permeability of cells to drugs and the effects of drugs on cell permeability must be elucidated ; the altered metabolism and reproduction of pathogen in the presence of drug must be interpreted in terms of cellular biochemistry. All these problems and more constitute the foundations for a science of chemotherapy ; at the present time we are only beginning to probe their nature. We are in the position of an aeroplane pilot flying above a fog bank, able to see some imposing peaks, but unable to do more than guess at the nature of the foundations of those peaks, or to find a way through the fog to a secure base. Our best approach to the foundation is to obtain as clear a picture as possible of the enzymic make-up of living cells, so that, eventually, we can reconstruct in exact chemical terms the series of events, which we call cell metabolism, by which life is carried on. The knowledge so gained should enable us to see through the fog hiding the foundation of our science, to appreciate the action of drugs on cell metabolism, and so to gain a secure base from which to conduct future operations in our search for chemotherapeutic substances.

Drugs as enzyme inhibitors

During the last twenty years considerable progress has been made in tracing a rough outline of cellular metabolism ; this progress has depended to a large extent on improved techniques which permit the isolation of pure enzymes. Even before the end of the nineteenth century, however, investigation of the crudest enzyme preparations had led to the recognition of the fundamental role of these biocatalysts in the living cell.

Preliminary work on enzymes, carried out between 1830 and 1840, is associated with the great names of chemistry such as Liebig, Wohler and Berzelius. Enzymes were then pictured as catalysts similar in action to inorganic catalysts, and any suggestion that enzymes were necessarily associated with life was strenuously opposed. The correct conclusions were only reached after much heated discussion, mostly based on yeast fermentation, between the chemists, led by Liebig, and the biologists, led by Pasteur. Liebig held that yeast was a catalyst formed by the action of atmospheric oxygen on non-living nitrogenous matter in fermentable liquids. Pasteur (1860) concluded that : " the chemical act of fermentation is essentially a phenomenon correlative with a vital act, commencing and ceasing with the latter. I am of the opinion that alcoholic fermentation never occurs without simultaneous organisation, development and multiplication of cells or the continued life of cells already formed. The results expressed in this memoir seem to be completely opposed to the opinions of Liebig and Berzelius. If I am asked in what consists the chemical act whereby the sugar is decomposed and what is the real cause, I admit that I am completely ignorant of it."

During the same year (1860) Berthelot showed that by maceration and washing of yeast, a cell-free preparation could be obtained which inverted cane sugar in the same way as did live yeast. The active principle could be precipitated by alcohol, washed and redissolved without loss of activity. He accordingly suggested that yeast acted on sugar by means of ferments which it was able to secrete. It is clear, he remarks, that the living cell itself is not the ferment but the producer of it.

Thereafter, more and more enzymes were identified and their activities correlated with those of the living cell. Finally, in the last quarter of the nineteenth century, we find that the enzymic nature of microbial action is fairly well established. Thus in 1899, Duclaux in his text book *Traité de Microbiologie*, when discussing the analogy between enzymes and microbes, says : " What one can do, the others can also, and the apparent strangeness of this identity of action between something living and something lifeless disappears partly when one learns that everything which we call the vital manifestation of a microbe occurs through the intermediation of an enzyme, which can be extracted and function outside of it. . . . Thus we can extract from it a substance which respires for the cell, another which digests its food, etc." Duclaux also reaches the interesting conclusion that the microbial cell does not differ essentially from " higher animals."

A logical sequence to the idea that enzymes are associated with microbial action is the investigation of the effect of antiseptics and drugs on enzymes. We find work on this subject as early as 1875, when Nasse investigated the effect of quinine, caffeine, strychnine and other alkaloids on yeast invertase, saliva and the pancreatic ferment ; he found considerable inhibition of invertase by quinine and strychnine. Hüfner (1874), wanting to find a means of preventing the development of micro-organisms in animal fluids without destroying soluble ferments, remarked that almost all substances capable of killing lower organisms destroy or inhibit the action of these ferments. Thereafter, a considerable amount of work was done on the inhibition of enzymes by antiseptics, although the results were unreliable, as the effect of *p*H on enzyme action was not then appreciated. Comparisons were made between the action of antiseptics on certain types of cell-free enzymes and on similar enzymes acting in the live bacterial cell ; thus, Fermi (1892) compared the action of thymol on trypsin and proteolytic bacteria. Duclaux (1899) realised that not all antiseptics act adversely on all enzymes, while some enzyme poisons do not act as antiseptics. Buchner, Buchner and Hahn (1903) noted that the inhibitory effect of antiseptics

on the fermentative powers of yeast juice was considerably less than their effect on fermentation by living cells.

After 1900, investigations gradually began to assume a more quantitative aspect. Hata (1909) investigated the effect of mercuric chloride, a powerful antiseptic, and showed that it inhibited many proteolytic enzymes but that inhibition could be reversed by substances, such as K_2S, which precipitate the mercury radical. This important observation on the reversible inactivation of enzymes by mercuric chloride is comparable with a much earlier observation that the disinfectant action of mercuric chloride could be antagonised by ammonium sulphide (Geppert, 1889).

Ehrlich, as a result of his early work on vital staining, was one of the first to stress that in cells there exist substances capable of oxidation and reduction which regulated the oxygen content of the cells. He termed these substances "chemoreceptors," but unfortunately appeared to ignore their relation to enzymes. However, by providing a theory of drug action based on direct chemical combination of drug with parasite, he stimulated the *in-vitro* study of the action of drugs on cells. Ehrlich's speculations were extended by Simon and Wood (1914) in the following terms: "Since intracellular metabolism is intimately connected with the action of enzymes, the question has naturally suggested itself whether the deleterious action of the dyes may not in part be referable to interference with the activity of these components." They suggest that the basic groups of inhibitory dyestuffs combine with acidic groups of "nutriceptors" of micro-organisms, and so inhibit the normal function of the "nutriceptors," which is to anchor and split foodstuffs. The cell dies, not necessarily because it has been poisoned, but because a sufficient number of nutriceptors have been thrown out of action to bring about its starvation or inability to multiply.

The suggestions of Ehrlich and of Simon and Wood were purely speculative, but they were soon given an experimental basis by Jacoby (1916) in a further study of the action of mercuric chloride. Jacoby found that the enzyme urease, which had been isolated from jack-bean and catalysed the

decomposition of urea to ammonia and carbon dioxide, was affected by mercuric chloride in the same way as were the bacteria which cause ammoniacal fermentation of urea. The concentration of mercuric chloride required to inhibit jack-bean urease was of the same order as that required to prevent ammoniacal fermentation by bacteria. The urea-splitting activity of the bacteria was, however, inhibited by concentrations of poisons which did not kill the bacteria. Inhibition of urease by mercuric chloride could be reversed by KCN. Jacoby, from a consideration of his results, suggested that three types of cell poisons could be distinguished :—

(1) Poisons acting by damaging gross cell structure.
(2) Poisons acting on reproduction and the synthesis of enzymes ; the growth of a bacterial culture being inhibited when the synthesis of its enzymes is prevented.
(3) Poisons acting directly by chemical combination with enzymes already present in the cell.

Jacoby's ideas differ little from those which we hold to-day, and experimental evidence has been slowly collected supporting and extending those ideas.

Among the pioneers, Rona in 1920 made a quantitative study of the effect of chemotherapeutic drugs on enzymes. Rigid control of *p*H provided reliable results for the action of quinine and its derivatives on invertase and serum lipase, and for seven arsenical drugs on urease and serum lipase (Rona and György, 1920 ; Rona and Petow, 1920 ; Rona and Bach, 1920 ; Rona and Bloch, 1921 ; Rona and Reinicke, 1921). The arsenicals provided interesting examples of specificity ; atoxyl and arsenic acid inhibited serum lipase but not urease, while methyl-arsenoxide inhibited urease but not lipase : atoxyl had no effect on lipase from guinea-pig serum, but was particularly active against human serum lipase. The toxicity of quinine and its derivatives was found to be directly dependent on *p*H, the results indicating that the free base is the toxic form of the drug.

The actual site of attack of arsenical drugs on enzymes

was suggested by some important work of Voegtlin, Dyer and Leonard (1923). The toxic effect on animals of the arsenoxide group was prevented by simultaneous injection of glutathione, a naturally-occurring polypeptide containing a free sulphydryl group. On the basis of the known chemical combination of arsenic with sulphydryl groups, the suggestion was made that arsenical drugs combine with sulphydryl groups in the cell, and inhibit essential metabolic processes associated with these groups. An excess of glutathione in the blood, or even of other sulphydryl-containing compounds such as thioglycols, can protect the essential cellular sulphydryl compounds. It is interesting to note that Ehrlich (1909) also suggested, but without experimental foundation, that sulphydryl groups could act as " chemo-receptors " for metals.

Investigations from 1913 until 1929 by Warburg and by Keilin included studies on the effect of poisons and narcotics on the respiration of intact organisms, and provided a much-needed link between isolated enzymes and cellular metabolism. In living intact cells, inhibition of respiration by a homologous series of narcotics, such as the urethanes or aliphatic alcohols, was found by Warburg (1914) to increase with increase in chain length, following lipoid/water distribution coefficients. When the same narcotics were added to cell-free enzyme preparations the homologous series rule also applied, but inhibition was less than in the intact cell ; Warburg suggested that this was due, at least in part, to the selective action of the cell membrane which transmitted lipoid-soluble substances rapidly to the interior of the cell. Certain lipoid-insoluble substances, such as barium chloride, had no inhibitory effect on intact cells, but acted as powerful poisons after damage of the cell wall. Cyanide, although it has an oil/water distribution coefficient of only 0·1, inhibited respiration at far lower concentration than narcotics and had a highly specific effect in preventing the uptake of oxygen. Warburg interpreted this effect as indicating the presence in cells of a cyanide-sensitive enzyme system (*Atmungsferment*) specially designed to activate molecular oxygen. The nature of this system, which even now is not fully understood, began to be elucidated after

Keilin (1925) had demonstrated the importance in living cells of iron porphyrin pigments to which he gave the name cytochrome. The oxidation of cytochrome in the living cell was found to be specifically inhibited by cyanide.

The specific action of cyanide was also demonstrated by Meyerhof (1917) in acetone-killed bacterial cells. Such cells had been shown by Cathcart and Hahn (1902) to reduce methylene blue in the absence of oxygen. Meyerhof found that inhibition of respiration by cyanide was much reduced by methylene blue, thus showing that the dye was taking the place of the cyanide-labile " Atmungsferment." The inhibition produced by narcotics was unchanged in the presence of methylene blue.

With the exception of one study on the inhibitory action of arsenic acid on cellular respiration (Onaka, 1910), little work on chemotherapeutic drugs was carried out by Warburg and his school, but the gap began to be filled in 1927 with the work of Quastel on the effect of antiseptics on metabolic processes of bacteria (Quastel and Wooldridge, 1927 *a* and *b*; Quastel and Wheatley, 1931; Quastel, 1931). The basis for this work was the fact that, in the presence of certain organic substances (known as substrates) which are broken down by cells, bacteria are capable of reducing anaerobically large quantities of methylene blue. This process Quastel attributed to " activation of the substrate " by specific enzymes of the organism. Exposure of bacteria to various antiseptics resulted in varying degrees of loss in capacity to oxidise individual substrates. Quastel's aim in this work is made clear in the statement . . . " Whether this experimental method will enable us to perceive precisely how an antiseptic exerts its lethal effect still remains to be seen, but it is clear that the method will allow us to compare and contrast the effects of certain lethal materials in a manner much more extensive than has hitherto been possible " (Quastel and Wooldridge, 1927). The toxicity of antiseptics, such as dyes, was not due to a general lethal action, since some activating mechanisms remained intact even after death of the cell, while specificity in action of dyes was also found—one dye inhibiting a certain

oxidation more than another. Exposure of *Escherichia coli* to copper sulphate, followed by washing, resulted in the elimination of many oxidising mechanisms which could be restored by treatment with hydrogen sulphide.

Quastel's next step was to obtain in cell-free state the enzymes (known as dehydrogenases) which oxidised individual substrates, and he compared the action of antiseptics on those enzymes with their action on the intact cell. He followed this with valuable work, which will be discussed in Chapter VII, on the effect of the structure of antiseptics on their enzyme-inhibitory properties.

. . . . . .

We have traced the growth of the idea that drugs act by inhibiting the normal enzymic reactions of the cell, and have now to see how far this theory can elucidate the mass of experimental data collected during the growth of chemotherapy. Before proof can be secured of the site of action of any particular drug, the enzymic processes which are assumed to be inhibited must be understood. At the time when Quastel was demonstrating the existence and inhibition of the dehydrogenating mechanisms of bacteria, the role of these dehydrogenases in metabolism was largely unknown. Since 1930 enzyme chemists have been concerned in elucidating the essential energy-yielding processes of the living cell; they have identified scores of enzymes and even obtained many in a highly purified crystalline state. The work has proceeded at such a pace that investigation of the action of drugs on enzymes has lagged behind the isolation of the enzymes themselves. Our best course, therefore, will be to outline in the rest of this chapter the present state of knowledge of the function of enzymes in intermediary metabolism, and in later chapters to interpret drug action in the light of this knowledge.

Nature of enzymes

All known enzymes are proteins with, so far as is known, much the same amino acid composition as other proteins. Many enzymes carry bound to the protein a relatively low molecular weight group, not linked by covalent bonds to the

rest of the molecule and not built up from amino acids on the normal protein-peptide pattern. This group in some enzymes is sufficiently firmly bound to be regarded as part of the enzyme molecule, and is in these cases usually referred to as a *prosthetic group*. In other cases, the union is reversible, and in solution the enzyme exists with this group (known then as the *coenzyme*) largely in the free state. Families of enzymes are known which have a common prosthetic group or coenzyme. Each member of such a family differs from the others in its specific protein which determines the nature of the reaction catalysed. It follows that the protein must possess some unique configuration capable of exerting a special influence on both substrate and prosthetic group. The exact nature of this influence remains a matter for speculation ; our ignorance on this point is covered by the statement that the substrate combines with, and is " activated " by, the " active centre " of the enzyme.

A fundamental problem faces any chemist investigating the reactions by which cells convert carbohydrates, fats or nitrogenous foodstuffs to water, to carbon dioxide or to the varying end products of cellular metabolism. Many of the substances involved are stable and most of the reactions involved cannot be carried out in the laboratory under physiological conditions. The biochemist therefore calls the useful word " activation " to his aid, and postulates that living cells have the ability to activate molecules through the use of enzymes. The exact physical forces involved in activation are unknown, but there is no doubt that enzymes, even when separated from the parent cells and transferred to the inhospitable environment of the chemical laboratory, can catalyse reactions with remarkable efficiency.

The energy liberated during the complete oxidation of a carbohydrate is considerable. The cell has evolved a whole series of integrated partial oxidations by which this energy is liberated or stored in small manageable packets ; these are utilised for " coupled " reductions of other foodstuffs or to force essential synthetic or endothermic reactions by which

vital substances are produced. These vital substances include the very enzymes involved in the activation, degradation and synthesis of intermediate metabolites; thus, an endless chain of events is involved in maintaining life. We recognise the existence of this chain but are still in the process of distinguishing the individual links.

Cytochrome enzymes

During respiration in air, living cells use up oxygen and produce mainly carbon dioxide and water as the end products of metabolism. To account for the formation of water it is necessary to find a system which, under physiological conditions, can catalyse the reaction $H_2+\frac{1}{2}O_2 \rightarrow H_2O$. This reaction occurs with explosive rapidity if the temperature is raised to 700°-800° C., but in the absence of catalyst cannot proceed at temperatures of 20°-37° C. We know that the rate of any reaction is determined by the frequency with which the reacting molecules may surmount an energy barrier; this barrier may be regarded as energy essential for rearrangement of the atoms in each molecule to form an unstable configuration, known as the "activated" form. At room temperature the number of molecules which can achieve this "activated" form is negligibly small in the case of hydrogen and oxygen, and, in absence of catalyst, reaction proceeds with immeasurable slowness. As the temperature is raised, the energy of each molecule is increased until a point is reached where a sufficient proportion of the molecules are "activated" for reaction to take place. In the presence of certain inorganic catalysts or of respiring cells, "activation" must also occur, since the reaction proceeds at considerable velocity.

The similarity between the inhibitory action of cyanide on cellular oxidation and on the aerobic oxidation of cysteine by artificial catalysts containing iron suggested to Warburg (1924) that the oxygen-activating system of living cells contained iron.

In 1925, during an examination of the thoracic muscles of certain insects, Keilin noted the existence of a pigment with characteristic absorption bands. The bands appeared when

the insect struggled violently or when it was deprived of oxygen, and disappeared when oxygen was present and the insect quiescent. Keilin concluded that the observed spectrum was that of the reduced form of a respiratory pigment which in the oxidised form had no characteristic absorption bands. Cyanide, which inhibited all respiration, also inhibited oxidation of the reduced form of pigment. Keilin was later able to show from spectroscopic studies that there were at least three such pigments which he named *cytochromes a, b* and *c*. The cytochromes are widely distributed, occurring in animals, plants and micro-organisms, and their absorption spectra (Fig. 1) indicate that all three are hæmochromogen pigments (Keilin, 1925, 1929, 1933).

The nature of cytochromes *a* and *b* is still unknown, as they have not been isolated, but they are probably mixtures of several components. Cytochrome *c*, which can be purified, has been found by Theorell to be a heat-stable hæmatin-protein of molecular weight 13,000; the porphyrin nucleus is firmly bound, probably through thioether linkages, to the protein. From degradative studies, Theorell has suggested the formula depicted in Fig. 2 (Theorell, 1938, 1941; Theorell and Åkesson, 1941).

Purified cytochrome *c* containing ferric iron can be reduced to the ferrous iron form by various reducing agents and re-oxidised by ferricyanide, but the reduced form is not oxidised at physiological *p*H by oxygen. In the living cell, reduced cytochrome *c* is readily re-oxidised in the presence of oxygen; hence there must be some system responsible for the physiological oxidation of reduced cytochrome. The nature of the enzyme which activates molecular oxygen and re-oxidises reduced cytochrome was indicated when Keilin found that cytochrome does not combine with cyanide or with carbon monoxide although both these poisons prevent its oxidation in the living cell. Carbon monoxide forms compounds with ferrous iron and particularly with iron porphyrins such as hæmoglobin; in the case of hæmoglobin such a compound is dissociated by light. Warburg (1926) showed that when the oxygen uptake of intact yeast cells was inhibited

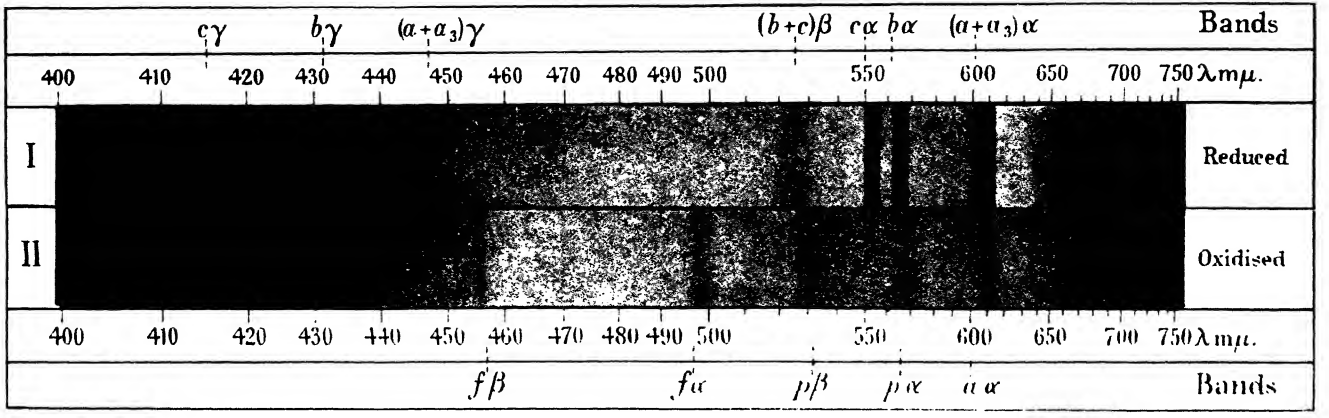


FIG. 1.—Diagrammatic figure of reconstructed absorption spectra of reduced and oxidised cytochrome in heart-muscle preparation. Spectrum II representing oxidised cytochrome, reconstructed from different depths of preparation, shows remains of reduced $a\alpha$-band, two diffuse parahæmatin bands ($p\alpha$ and $p\beta$) of compounds *b* and *c*, and two diffuse bands (f) of a flavoprotein compound, which become hardly perceptible in preparations showing reduced cytochrome. (For full details see Keilin and Hartree, 1939.)

by carbon monoxide and the poisoned cells were then exposed to light of suitable wavelength, respiration was resumed. The effectiveness of light in reversing the carbon monoxide inhibition varied with the wavelength used. If the absorption coefficient (*i.e.* the effectiveness of the light in restoring

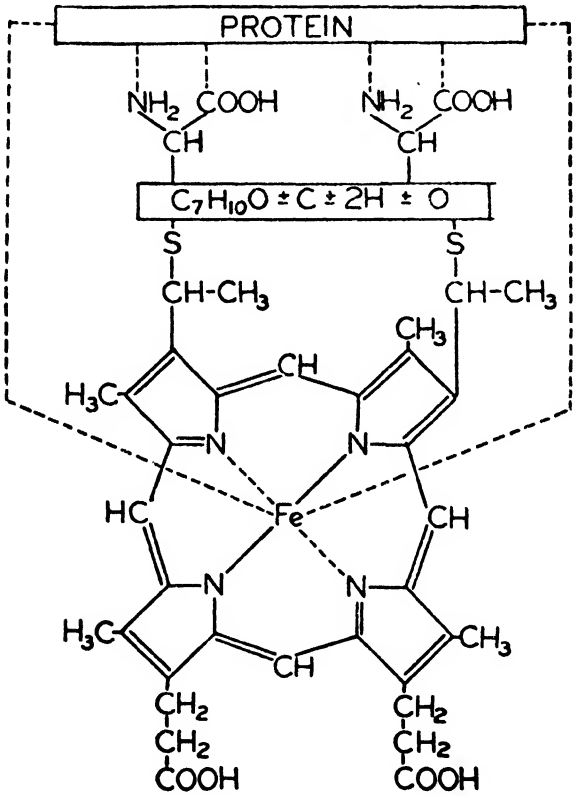


FIG. 2.—Structure of cytochrome c. (Theorell, 1941.)

respiration) was plotted against wavelength, a photochemical absorption curve was obtained closely similar to that of carboxy-hæmoglobin (Warburg and Negelein, 1928). Warburg accordingly suggested that hæmin iron in the form of a thermolabile enzyme (the *Atmungsferment*) played a vital part in respiration. Warburg's *Atmungsferment* is inhibited by

cyanide and by carbon monoxide and is thermolabile; the cellular system which catalyses the oxidation of reduced cytochrome *c* is also thermolabile, but cytochrome *c* itself is thermostable. These facts suggested to Keilin that the oxygen-activating enzyme (*Atmungsferment*) is responsible for the oxidation of reduced cytochrome by molecular oxygen in the living cell (Keilin, 1929). The enzyme was renamed

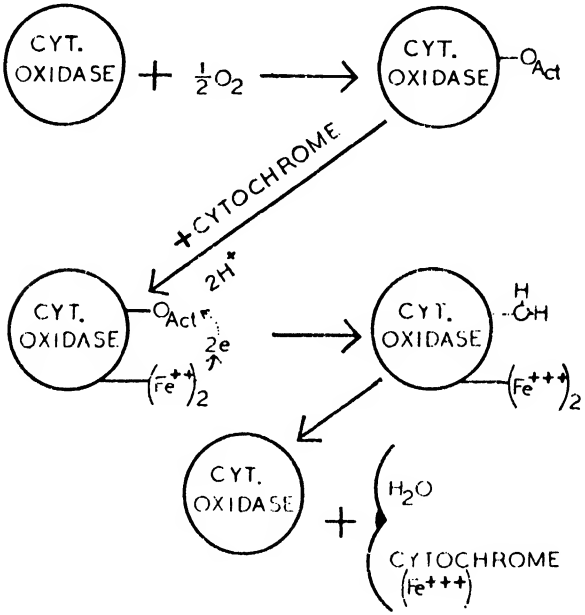


FIG. 3.—Aerobic oxidation; cytochrome system. (Oxidised cytochrome is represented by Fe^{+++}, reduced cytochrome is represented by Fe^{++}, electron transfer is represented by e⋯→.)

cytochrome oxidase. Up to the present, all attempts to purify cytochrome oxidase have failed; even the preparation of a cell-free solution has proved difficult. Claims have been made for the success of a method involving supersonic disintegration of cell structure (Haas, 1943), but these are doubted by Keilin and Hartree (1947). Extraction with sodium desoxycholate has been said to produce a soluble preparation with high activity (Wainio, Cooperstein, Kollen

and Eichel, 1947). The spectrum of cytochrome oxidase has been provisionally identified by direct spectroscopy (Keilin and Hartree, 1939).

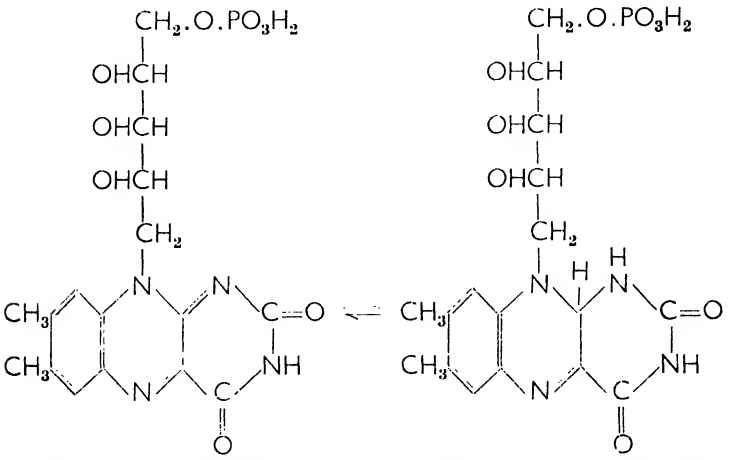
If we represent enzymic protein by a circle, "activated" oxygen by the symbol O_{Act} and the oxidised and reduced forms of cytochrome as (Fe^{+++}) and (Fe^{++}) respectively, the oxidation of reduced cytochrome in cellular metabolism can be depicted diagrammatically as in Fig. 3. The representation of the activated complex as containing 2 mols. of cytochrome is not intended to imply that the reaction is necessarily trimolecular. The source of the protons (H^+) will become evident when we extend our discussion to the biological mechanism for reduction of cytochrome to the (Fe^{++}) form.

Flavoprotein enzymes

Keilin showed by spectroscopic observation that in living cells cytochrome is reversibly reduced and oxidised, and, with cytochrome oxidase, provides a mechanism for the participation of molecular oxygen in respiration. If purified cytochrome *c* is reduced chemically and added to cytochrome oxidase preparations, in the presence of oxygen it is rapidly converted to the oxidised form, but in the absence of oxygen is not re-reduced, as it is in the living cell. The living cell must therefore possess a mechanism for the reduction of cytochrome *c*. A whole group of respiratory enzymes, known as flavoproteins, has been isolated and crystallised; some of these probably provide a mechanism for the *in-vivo* reduction of the cytochromes and can be used *in vitro* to reconstruct artificial systems in which the oxidation-reduction cycles of living cells are simulated.

The first flavoprotein to be isolated was the "old yellow enzyme," discovered by Warburg and Christian (1932) and later crystallised by Theorell (1935). By dialysis against dilute acid, the enzyme can be split into a soluble yellow prosthetic group, and a colourless protein which is insoluble in acid but redissolves on neutralisation. On addition of the yellow dialysate to the neutralised protein solution, the enzyme is rapidly reconstituted. The yellow prosthetic group

has been identified as alloxazine mononucleotide, the phosphoric ester of riboflavin, a member of the vitamin B complex (Review : Theorell, 1937).



Alloxazine mononucleotide *Dihydro-alloxazine mononucleotide (leuco form)*

The yellow solution of riboflavin phosphate is readily reduced by hyposulphite to the colourless " leuco " form, as indicated in the formulæ above. The " leuco " flavin is reoxidised to the yellow quinonoid form by shaking with air. The complete flavoprotein behaves in the same way and thus might act as a cellular catalyst for the transfer of hydrogen to molecular oxygen. However, the oxidation of the reduced enzyme in air is so slow that it is unlikely to be of much use to the cell and cannot account for more than a small fraction of the observed oxygen uptake of aerobic cells.

The " old yellow enzyme " is incapable of reducing cytochrome, but two other flavoproteins have been isolated which probably act as cellular reducing agents for the cytochrome system. *Cytochrome reductase*, isolated from yeast by Haas, Horecker and Hogness (1940), has the same molecular weight as the old yellow enzyme (78,000) and the same prosthetic group, but has quite distinct catalytic properties. The reduced " leuco " form of cytochrome reductase is not oxidised by molecular oxygen ; but, if added to a solution

of cytochrome *c*, it is rapidly converted to the oxidised form, while cytochrome *c* is simultaneously reduced. The three proteins cytochrome reductase, cytochrome *c* and cytochrome oxidase therefore provide a limited system for the catalysis

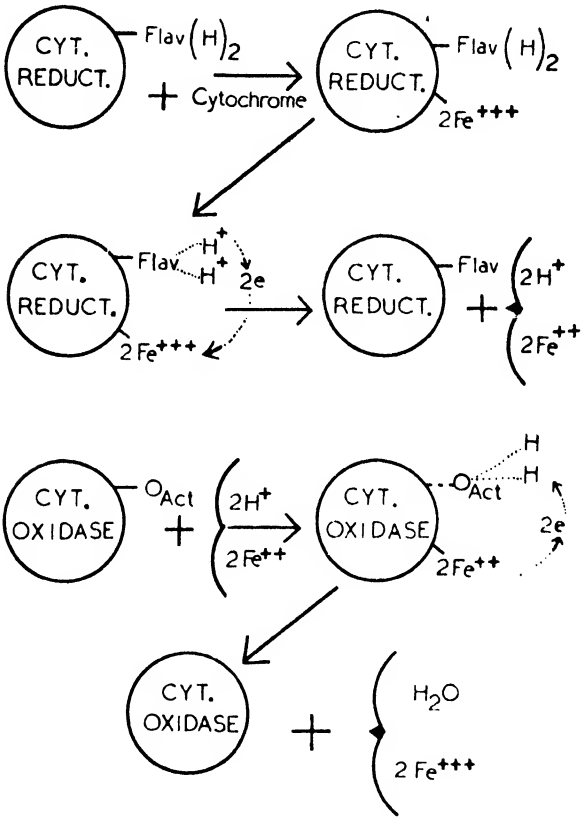
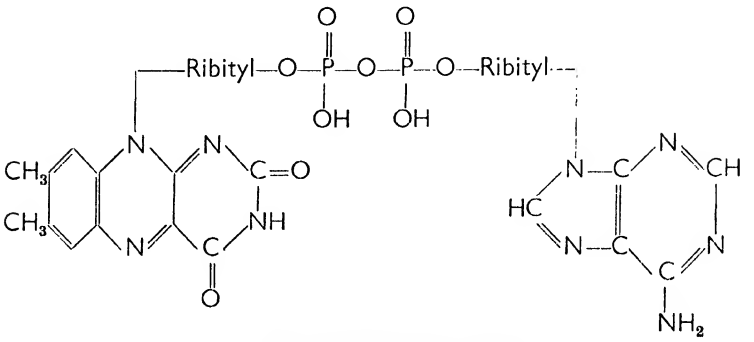


FIG. 4.—Aerobic oxidation; flavoprotein-cytochrome system. (Oxidised cytochrome is represented by Fe^{+++}, reduced cytochrome is represented by Fe^{++}, cytochrome reductase is abbreviated to CYT. REDUCT.)

of the reaction $H_2+\frac{1}{2}O_2 \rightarrow H_2O$. The series of reactions involved is represented diagrammatically in Fig. 4, the reduced and oxidised forms of riboflavin being represented as —Flav H_2 and —Flav; other abbreviations are as in Fig. 3.

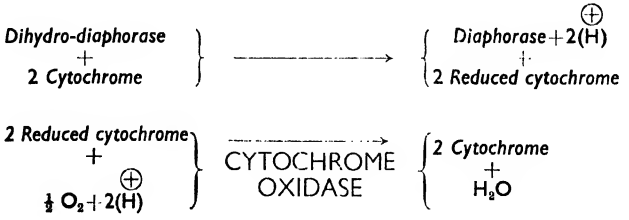
The second yellow enzyme which is believed to act through the cytochrome system is known as *diaphorase* and was

purified by Straub (1939). Its prosthetic group was identified as alloxazine adenine dinucleotide.



Alloxazine adenine dinucleotide

Diaphorase, like cytochrome reductase, can be reduced to a colourless leuco-form which does not re-oxidise on shaking with air, but reduced diaphorase fails to react with cytochrome *c*. When added to the complete cytochrome-cytochrome oxidase system, reduced diaphorase is, however, extremely rapidly oxidised by a cyanide-sensitive heat-labile enzyme system, which is presumably one of the group of *a* or *b* cytochromes. The transfer of hydrogen from the dihydro-iso-alloxazine group of reduced diaphorase to molecular oxygen may be represented by the following linked series of reactions.



These flavin enzymes are widely distributed in the cells of animals and micro-organisms and, with the cytochrome system, provide a catalytic mechanism for the participation of oxygen in cellular metabolism under physiological conditions. If this system is to work, the cell must also possess methods

for reducing the flavin enzymes. It will be remembered how Quastel showed that bacteria were capable of dehydrogenating various normal metabolites anaerobically in the presence of methylene blue, which acted as a hydrogen acceptor. A whole group of enzymes, the *pyridine nucleotide dehydrogenases*, have since been identified as specific catalysts for the dehydrogenation of such metabolites. These dehydrogenases in turn reduce diaphorase or cytochrome reductase and thus act as catalysts for the aerobic oxidation of cellular metabolites.

Pyridine nucleotide dehydrogenases

We have already remarked on the controversy carried on for about twenty years between the adherents of the theories of Liebig and of Pasteur on the nature of enzymes. Much of the support for Pasteur's belief in the " vital " origin of ferments depended on the fact that all early experiments designed to produce fermentation in the absence of living cells had failed. Berthelot, it will be remembered, expressed the view that organisms were not themselves ferments but rather the producers of ferments, but he had been unable to ferment sugar and produce carbon dioxide and alcohol in the absence of living cells. A method of preparation from yeast of a cell-free filtrate capable of fermenting sugar was discovered by Buchner (1897). Buchner concluded that " the production of alcoholic fermentation does not require so complicated an apparatus as the yeast cell, and that the fermentative power of yeast-juice is due to the presence of a dissolved substance—Zymase." Harden and Young (1906) showed that Buchner's juice could be separated into a thermolabile protein component and a thermostable dialysable component to which they gave the name *Co-zymase*. Neither component was active by itself, but together they constituted the whole activity of zymase.

The nature and function of Co-zymase, or as it was later called, *coenzyme I*, remained something of a mystery for more than twenty years. From 1925 to 1935 methods were gradually worked out for its purification, and it was finally identified as

a pyridine adenine dinucleotide with the structure shown in Fig. 5A (Euler and Schlenk, 1937).

While the work on coenzyme I was still incomplete, Warburg and Christian (1931) discovered a different thermostable coenzyme in red blood cells. They found that laked

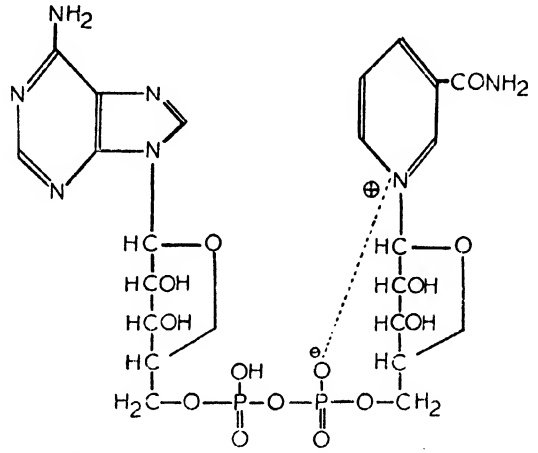


FIG. 5A.—Coenzyme I (diphosphopyridine nucleotide).

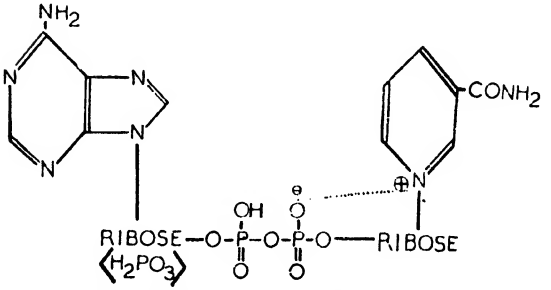
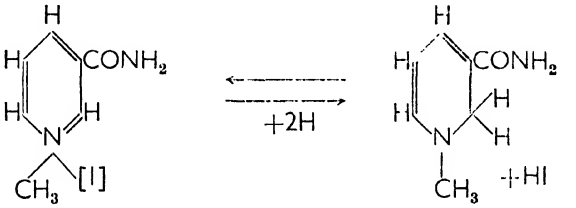


FIG. 5B.—Coenzyme II (triphosphopyridine nucleotide).

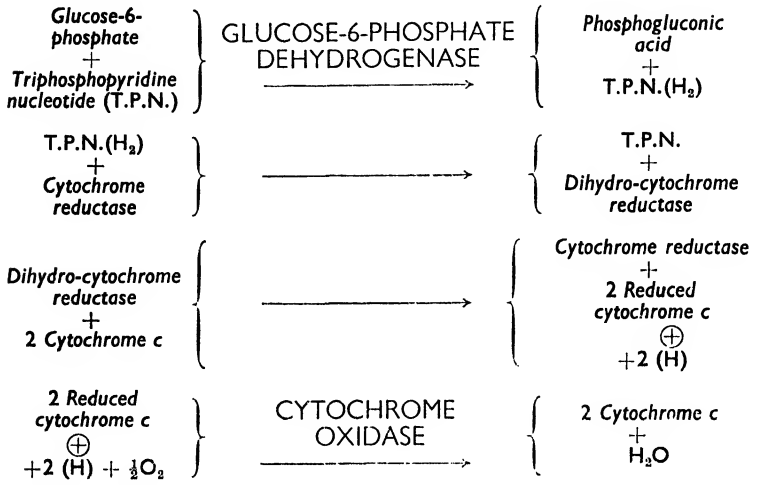
horse erythrocytes gave an enzyme system capable of oxidation of glucose-6-phosphate, and separated the system into a thermolabile enzyme and a thermostable substance, *coenzyme II*, which resembled, but was not replaceable by, coenzyme I. Warburg isolated coenzyme II, determined its structure (triphosphopyridine nucleotide, Fig. 5B) and elucidated its

mode of action (Warburg, Christian and Griese, 1935). Both diphosphopyridine nucleotide and triphosphopyridine nucleotide have characteristic absorption spectra, and both undergo a similar change in spectrum when reduced with alkaline hyposulphite. Reduction involves the uptake of two hydrogen atoms ; its nature was indicated when nicotinamide methiodide was shown to undergo a similar reduction with similar change in absorption spectrum (Karrer, Kahnt, Epstein, Jaffé and Ishii, 1938 ; Karrer, Ishii, Kahnt and van Bergen, 1938). This reduction of the methiodide can be represented as follows :



Warburg found that coenzyme II showed a change in absorption spectrum when added to a solution of red cell enzyme + glucose-6-phosphate ; in other words, an enzyme existed in blood corpuscles capable of catalysing the transfer of hydrogen from glucose-6-phosphate to triphosphopyridine nucleotide, so bringing about the oxidation of glucose-6-phosphate. The enzyme, known as glucose-phosphate dehydrogenase, was also found to occur in yeast (Warburg, Christian and Griese, 1935 ; Negelein and Haas, 1935 ; Negelein and Gerischer, 1936). If purified glucose-6-phosphate dehydrogenase is added to a solution of glucose-6-phosphate and triphosphopyridine nucleotide, glucose-6-phosphate is converted to phosphogluconic acid only in so far as there is unreduced triphosphopyridine nucleotide available to act as hydrogen acceptor. Dihydro-triphosphopyridine nucleotide is not capable of transferring hydrogen directly to molecular oxygen, but requires cytochrome reductase to accept its hydrogen. In fact, the two flavin enzymes, diaphorase and cytochrome reductase, act as specific catalysts for the transfer of hydrogen from reduced coenzymes I and II through the cytochrome system to oxygen. The system for the oxidation

of glucose-6-phosphate to phosphogluconic acid and water may then be represented diagrammatically as follows :—



Glucose-6-phosphate dehydrogenase is only one of a large group of enzymes which catalyse the transfer of hydrogen from metabolite to one of the pyridine nucleotides. Many of these enzymes, known collectively as the pyridine nucleotide dehydrogenases, have been isolated in crystalline form (see review, Schlenk, 1945). By activating metabolites they provide the first link in a chain of oxidation-reduction reactions which results in a stepwise transfer of hydrogen from cellular metabolite to molecular oxygen as indicated in the above scheme. They do much more than this however, since they function in anaerobic as well as in aerobic metabolism.

We have already noted that under anaerobic conditions, but in the presence of methylene blue, both animal and bacterial cells can catalyse the oxidation of metabolites with simultaneous reduction of methylene blue to the "leuco" form. Living anaerobic cells obviously cannot utilise methylene blue as their biological hydrogen acceptor. Nevertheless, in a closed anaerobic system, each oxidative step must be accompanied by a corresponding reduction, and cells capable of anaerobic metabolism must possess means for the removal of hydrogen from reduced coenzymes. That the cytochrome

system plays no part in these "linked" oxidation-reductions is indicated by the absence of any cytochrome absorption bands in suspensions of certain bacteria. Bacteria may be classed as aerobes, facultative anaerobes (which can utilise oxygen but may be able to grow in its absence) and strict anaerobes (which only grow when oxygen is completely excluded). The accompanying Table 1 shows the occurrence of cytochrome in some bacteria in each of these classes. Among the facultative anaerobes one or more of the absorption bands of the cytochrome complex is often found to be missing, while in the strict anaerobes no cytochrome bands are visible.

TABLE 1

Distribution of cytochromes in bacteria

Organism	Respiratory Character	Cytochromes
B. anthracis	Aerobe	Present
M. tuberculosis	Aerobe	Present
S. aureus	Aerobe	Present
V. cholerœ	Aerobe	Present
E. typhosa	Facultative anaerobe	Partially present
S. dysenteriæ	Facultative anaerobe	Partially present
L. delbrückii	Facultative anaerobe	Absent
Cl. welchii	Anaerobe	Absent
Cl. tetani	Anaerobe	Absent

(Adapted from Stephenson, 1939)

The presence of pyridine nucleotides and their dehydrogenases in both aerobic and anaerobic cells, coupled with the absence of the cytochrome system from anaerobic cells, suggests similarity in the metabolic pathways in both types of cells up to a common point; beyond this point in aerobic cells the cytochrome system evidently takes over. As we have seen, aerobic cells contain two important flavoprotein enzymes which are specifically designed to convert the dihydro-pyridine nucleotides to the oxidised form through the cytochrome system, so making the two coenzymes available for further reaction with dehydrogenase and metabolite. Anaerobic cells must achieve the same re-oxidation of dihydro-pyridine nucleotides by other means. An example of such a mechanism is provided by the dehydrogenase which catalyses the dehydrogenation

of alcohol to acetaldehyde. This enzyme has been isolated from yeast cells in crystalline form, and requires diphosphopyridine nucleotide as a coenzyme (Negelein and Wulff, 1937). The reaction involved in the oxidation of alcohol can be represented as

$$\left.\begin{matrix}\text{Ethyl alcohol}\\ +\\ \text{D.P.N.}\end{matrix}\right\} \xrightleftharpoons{\text{ALCOHOL DEHYDROGENASE}} \left\{\begin{matrix}\text{Acetaldehyde}\\ +\\ \text{D.P.N.}(H_2)\end{matrix}\right.$$

Like most enzymic reactions, the reaction is reversible; the direction depends on the amounts of the reactants present and

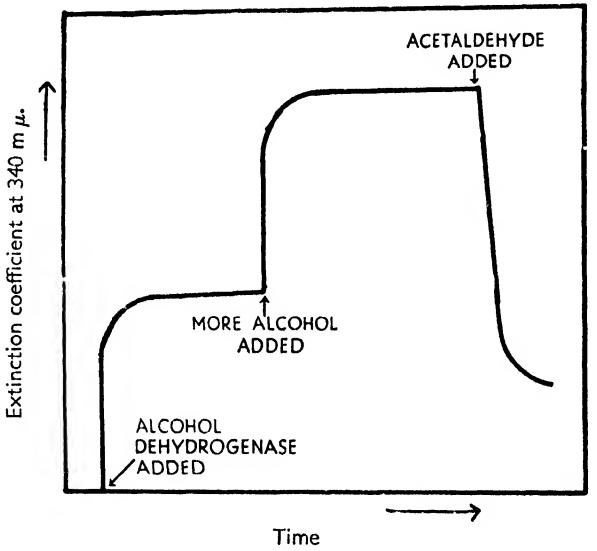


FIG. 6. — Reversible oxidation and reduction of diphosphopyridine nucleotide by alcohol dehydrogenase in the presence of alcohol or acetaldehyde. Starting mixture contains diphosphopyridine nucleotide and alcohol. (Adapted from Euler, Adler and Helstrom, 1936.)

on external conditions such as *p*H and temperature. The course of the reaction in an isolated and purified preparation can be readily followed spectrophotometrically since the reduced form of coenzyme has a distinct absorption band at 340 mμ which disappears on oxidation. If purified dehydrogenase, alcohol and excess diphosphopyridine nucleotide are mixed and the change in extinction coefficient with time is plotted (Fig. 6), a curve is obtained representing the degree

of conversion of nucleotide to dihydro-nucleotide. The point where the curve flattens represents the equilibrium point for the original mixture. If, after equilibrium has been reached, more alcohol is added to the system, the extinction coefficient is increased, thus indicating further conversion of nucleotide to dihydro-nucleotide; if aldehyde is added the opposite effect is observed.

The equilibrium constant (K) for the reaction may be calculated from such spectrophotometric data (Negelein and Wulff, 1937). According to the law of mass action,

$$\frac{[\text{D.P.N.}]}{[\text{D.P.N.H}_2]} \frac{[\text{Alcohol}]}{[\text{Aldehyde}]} = \text{K}$$

(concentration of reactants is indicated by [ ]). At *p*H 7·0 and 38°, K was calculated to be $2{\cdot}14 \times 10^4$. This means that when the coenzyme is half reduced, *i.e.* when

$[\text{D.P.N.}] = [\text{D.P.N.H}_2]$, the value $\frac{[\text{Alcohol}]}{[\text{Aldehyde}]} = 2{\cdot}14 \times 10^4$

In other words the equilibrium is greatly in favour of accumulation of alcohol, a concentration of $2{\cdot}14 \times 10^4$ mols. of alcohol per mol. of aldehyde being possible. Under physiological conditions, therefore, the reaction will proceed in the direction of alcohol formation with consequent conversion of reduced pyridine nucleotide to the oxidised form. Acetaldehyde, as we shall see, is one of the main intermediary products of anaerobic fermentation; its reduction to alcohol thus provides a method for the anaerobic removal of hydrogen from dihydro-diphosphopyridine nucleotide. What is in reality occurring is an oxidation of one substrate coupled with simultaneous reduction of another.

Metabolism of carbohydrate brought about in the absence of oxygen is called *fermentation*, the particular type of fermentation taking its name from the predominant end-product; thus, alcoholic fermentation, acetic acid fermentation, propionic acid fermentation, acetone fermentation, lactic acid fermentation, butyric acid fermentation, and so on.

The question might well be asked, what does the cell gain by fermentation? Carbohydrate seems to be the main

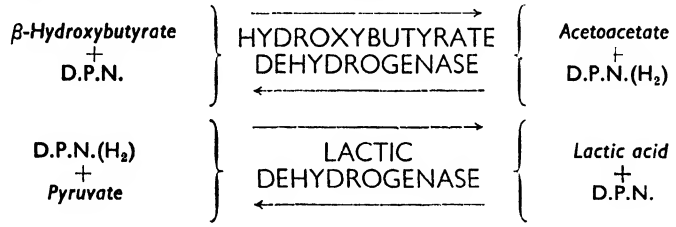
energy-provider of metabolism, the free energy change in the complete oxidation of glucose to carbon dioxide and water being 686 kg. cals./mol. In the absence of oxygen, the cell is forced to obtain energy by carrying out either molecular rearrangements which involve gain in free energy, or coupled reactions in which energy released by one change is utilised to force another reaction. The overall energy yield in these cases is very much less than in total oxidation ; ethyl alcohol fermentation makes available only 7·9 per cent. of the total energy of aerobic oxidation, while the most efficient fermentation, that giving rise to propionic acid, only yields 79 kg. cals. per gram mol. The rest of the potential energy of combustion remains unused in the waste products of fermentation. This great waste of energy is probably compensated for by the independence of oxygen supply which the cell gains through adopting this mode of life.

Energy transfer and organic phosphate

Many micro-organisms are able to grow on media consisting of inorganic salts plus a single organic compound such as glucose, glycerol or acetic acid. During growth, protein, polysaccharide, fat and all the various complex organic molecules which go to make up the living cell are synthesised. Many reactions of these synthetic processes involve an increase in free energy, and may only occur if coupled in some way with reactions able to supply the necessary energy. The nature of the energetic coupling between energy-liberating (*exergonic*) reactions and energy-consuming (*endergonic*) reactions is therefore a fundamental problem of cellular metabolism (Fruton, Ball, Bergmann, Kalckar, Meyerhof and Smythe, 1944 ; Lipmann, 1946*b*). It is incidentally of considerable interest in any study of the mode of action of drugs, since certain cell poisons inhibit cell synthesis without inhibiting oxidation (Clifton and Logan, 1939 ; Hotchkiss, 1944 ; Spiegelman and Kamen, 1946).

One method of energetic coupling has already been mentioned, linked oxidation-reduction. In the reduction of pyruvic acid to lactic acid there is an increase in the free

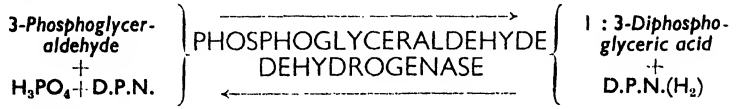
energy of the system. Such an endergonic reaction is thermodynamically impossible without a source of energy. This is the case even in the presence of an enzyme, since by definition, an enzyme can only catalyse a thermodynamically-possible reaction. Nevertheless lactic acid is an end-product derived from pyruvic acid in fermentation of sugars. The possibility of energetic coupling of pyruvic acid reduction with oxidation of β-hydroxybutyric acid (an exergonic reaction) has been shown by Green, Dewan and Leloir (1937). Pyridine nucleotide dehydrogenases are catalysts for both reactions, and the common coenzyme I links them. The oxidation of β-hydroxybutyrate provides both the energy and the hydrogen necessary for the reduction of pyruvate, and the formation of lactate is made possible because the overall energy change of the system is negative (ΔF is negative). The reactions involved can be represented diagrammatically as follows :—



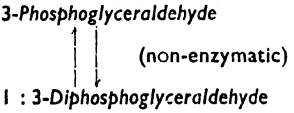
Phosphorylation is another process which is intimately connected with energy transfer in cell metabolism. We have mentioned that lysed red cells contain the enzyme glucose-6-phosphate dehydrogenase, which in the presence of coenzyme II oxidises glucose-6-phosphate to phospho-gluconic acid. The dehydrogenase cannot oxidise glucose itself, although intact red cells do so. Such a result implies that the cells possess, in addition to a system for the oxidation of glucose-6-phosphate, an enzyme capable of converting glucose to glucose-6-phosphate. The reaction glucose+phosphoric acid→glucose-6-phosphate is endergonic ; the intact cell must therefore possess a means of transferring energy from an energy-yielding reaction in order to bring about the phosphorylation of glucose.

Warburg and Christian (1939) were able to demonstrate

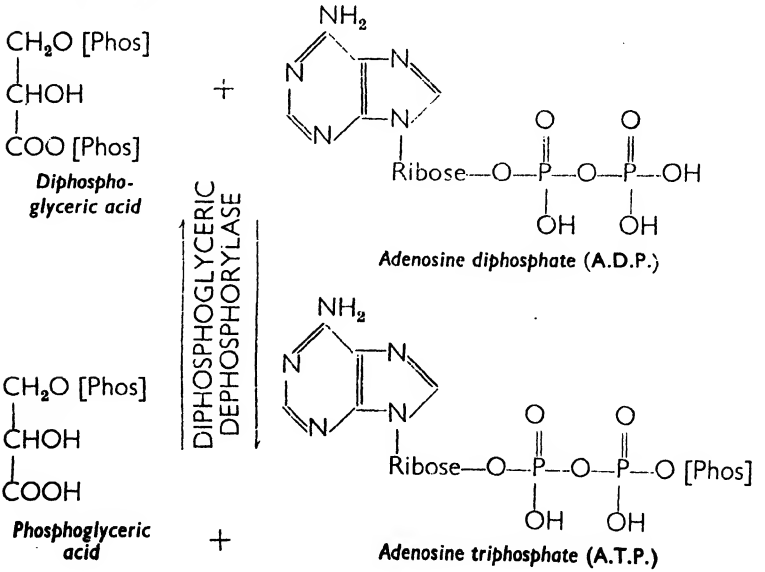
coupling of an exergonic oxidative reaction with an endergonic uptake of inorganic phosphate. The phosphate, by a mechanism of phosphate transfer, was made available for the phosphorylation of hexose. Phosphoglyceraldehyde, in the presence of inorganic phosphate and its specific dehydrogenase is oxidised and phosphorylated to diphosphoglyceric acid. Diphosphopyridine nucleotide acts as coenzyme and hydrogen acceptor. The exact mechanism of phosphate uptake is not



quite clear; Warburg suggests that 3-phosphoglyceraldehyde forms a loose addition compound with phosphoric acid prior to oxidation. Diphosphoglyceric acid, in the presence of adenosine



diphosphate and a specific dephosphorylating enzyme (crystallised by Bücher, 1942), is transformed to monophosphoglyceric acid, the phosphate group bring transferred to adenosine diphosphate as indicated in the following scheme :—



The formation of adenosine triphosphate from adenosine diphosphate and phosphoric acid is endergonic, so also is the formation of diphosphoglyceric acid from phosphoglyceric acid and phosphoric acid. The necessary energy for the uptake of inorganic phosphate is supplied by the oxidation of aldehyde to acid and this energy is transferred to adenosine diphosphate with transfer of the phosphate group. Adenosine triphosphate may then be considered to contain an "*energy-rich*" phosphate bond.

An enzyme, hexokinase, has also been purified and crystallised, which, in the presence of a thermostable coenzyme identified as adenosine triphosphate, is capable of catalysing the phosphorylation of glucose (Berger, Slein, Colowick and Cori, 1946; Kunitz and McDonald, 1946). As already

$$\textit{Glucose} + \text{A.T.P.} \xrightarrow{\text{HEXOKINASE}} \textit{Glucose-6-phosphate} + \text{A.D.P.}$$

mentioned, the phosphorylation of glucose by inorganic phosphate is an endergonic reaction but, in the presence of hexokinase and adenosine triphosphate, glucose is phosphorylated with simultaneous evolution of heat. The energy for the reaction is provided by the energy-rich phosphate of adenosine triphosphate which is transferred to glucose, while adenosine triphosphate is converted to adenosine diphosphate.

There occur in the living cell various organic compounds of phosphorus in which it is possible to distinguish two types of phosphate bond (Lipmann, 1941; Kalckar, 1941). Where phosphoric acid is linked to an alcoholic hydroxyl, as in the sugar phosphates, the free energy change on hydrolysis is only of the order of 1 to 3 kg. cals. per mol. If phosphoric acid is linked to nitrogen as in creatine phosphate, to a carboxyl group as in diphosphoglyceric acid, or to another phosphate group as in adenosine triphosphate, the free energy change on hydrolysis is very much greater, being of the order of 8-10 kg. cals. per mol. These compounds contain the energy-rich phosphate bonds referred to previously. Adenosine triphosphate appears to function as a carrier and reserve of energy-rich phosphate bonds, and by its intervention

as a coenzyme many endergonic synthetic reactions become possible. The adenosine diphosphate formed in these reactions is then reconverted to adenosine triphosphate by exergonic reactions involving formation of further energy-rich phosphate bonds. Here we have the "manageable packets of energy," which, earlier in this chapter, we put forward as the mechanism adopted by the cell for storage or transfer of energy. The energy transferred with one energy-rich phosphate bond amounts to about one-fiftieth of that liberated by total oxidation of one carbohydrate molecule.

As we consider in detail the metabolism of cells and the interplay of oxidation-reduction and phosphorylation in this metabolism, the methods adopted in the living cell for the accomplishment of synthetic reactions should become more fully evident.

Anaerobic carbohydrate breakdown

Preliminary phosphorylation appears to be essential for the greater part of anaerobic breakdown of carbohydrate. The phosphorylation, if endergonic, may be carried out at the expense of energy derived from later stages, adenosine triphosphate acting as the energy- and phosphate-carrier by means of its energy-rich phosphate bonds. Whatever the type of carbohydrate, whether polysaccharide, disaccharide or monosaccharide, it can be transformed into fructose diphosphate before being degraded. In the case of glucose, the first stage has already been discussed, conversion by the enzyme hexokinase to glucose-6-phosphate. The enzyme phosphohexose isomerase (Meyerhof and Beck, 1944) catalyses the next step, rearrangement of glucose-6-phosphate to fructose-6-phosphate (see Fig. 7). A second phosphate group, also derived from adenosine triphosphate, is then introduced by the enzyme phosphohexokinase. The reaction product, fructose diphosphate, thus requires for formation from glucose two energy-rich phosphate bonds which must be provided in the form of adenosine triphosphate derived through linked exergonic reactions.

Fructose diphosphate is split by the enzyme zymohexase

(Herbert, Gordon, Subrahmanyan and Green, 1940) and forms glyceraldehyde phosphate and dihydroxyacetone phosphate. Dihydroxyacetone phosphate is not further degraded as such, but is immediately rearranged by the enzyme isomerase to glyceraldehyde phosphate with the nett result that two molecules of glyceraldehyde phosphate are formed from one of fructose diphosphate. The series of reactions up to this point may be represented conveniently as in Fig. 7 in the form used by Lipmann (1941).

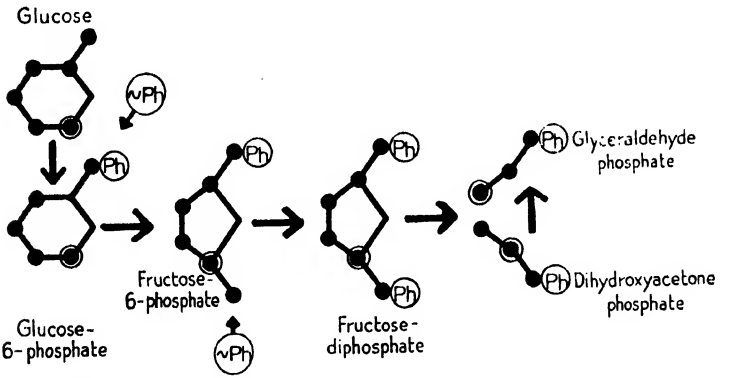
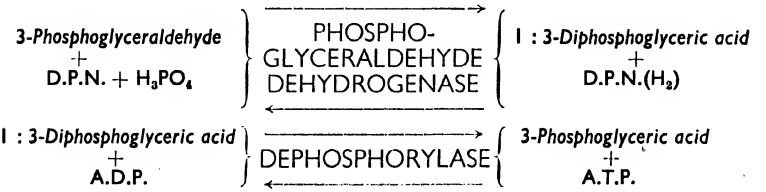


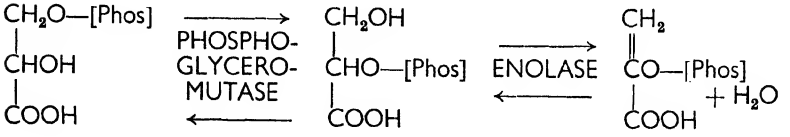
FIG. 7.—Anaerobic breakdown of glucose to triosephosphate [~Ph = energy-rich phosphate bond]. (Lipmann, 1941).

The oxidation of phosphoglyceraldehyde to 3-phosphoglyceric acid with generation of an energy-rich phosphate bond has already been described (p. 66). It can be summarised as follows :—



The adenosine triphosphate formed at this stage is available for the phosphorylation of a further molecule of hexose. The system described obviously provides for a repetitive cycle of events by which endergonic reactions can proceed at the expense of linked exergonic reactions.

3-Phosphoglyceric acid is not an end-product of anaerobic metabolism, but is further transformed by the enzyme phosphoglyceromutase to 2-phosphoglyceric acid, and this compound with the enzyme enolase is dehydrated to phosphoenolpyruvate.



The intra-molecular rearrangement of 3-phosphoglyceric acid has created in phosphoenolpyruvate an energy-rich phosphate bond. Once more, adenosine diphosphate is a recipient of this energy, forming adenosine triphosphate and pyruvic acid under the influence of a dephosphoryling enzyme. The adenosine triphosphate is then available as a source of energy to force other desirable endergonic reactions.

The production of two molecules of pyruvic acid from one molecule of hexose has involved utilisation of two energy-rich phosphate bonds and formation of four of these bonds, so that, overall, two energy-rich phosphate bonds are generated by this anaerobic phase of carbohydrate breakdown. It will perhaps be helpful at this point to draw up a table of the reactions involved (Table 2). The enzymes concerned are also listed; a number of them have been isolated, purified and crystallised, and several have been found to be metalloproteins.

Phosphorylative breakdown of carbohydrates has been shown to occur in trypanosomes and malaria parasites, as well as in yeast and bacteria (Chen and Geiling, 1946; Speck and Evans, 1945*a*; Evans, 1946). It is therefore probable that the various reactions described in Table 2 are common to the majority of unicellular organisms as well as to higher animals, but they do not necessarily represent the *only* available pathway for carbohydrate breakdown. Pyruvic acid is, however,

TABLE 2

Anaerobic breakdown of glucose

Reaction	Enzyme	Isolation of Enzyme
Glucose + A.T.P. ↓ Glucose-6-phosphate + A.D.P.	Hexokinase	Metalloprotein (magnesium). Crystallised by Kunitz and Macdonald (1946), and by Berger, Slein, Colowick and Cori (1946)
Glucose-6-phosphate ⇅ Fructose-6-phosphate	Phosphohexose isomerase	Partially purified by Meyerhof and Beck (1944)
Fructose-6-phosphate + A.T.P. ⇅ Fructose-1 : 6-diphosphate + A.D.P.	Phosphohexokinase	Not purified
Fructose-1 : 6-diphosphate ⇅ Glyceraldehyde-3-phosphate + dihydroxyacetone phosphate	Zymohexase (Aldolase)	Metalloprotein (zinc, iron, cobalt, or copper). Crystallised by Warburg and Christian (1943)
Dihydroxyacetone phosphate ⇅ Glyceraldehyde phosphate	Triosephosphate isomerase	Purified by Meyerhof and Beck (1944)
1 : 3-Diphosphoglyceraldehyde + D.P.N. ⇅ 1 : 3-Diphosphoglyceric acid + D.P.N.(H_2)	Phosphoglyceraldehyde dehydrogenase	Crystallised by Warburg and Christian (1939), and by Cori, Slein and Cori (1945)
1 : 3-Diphosphoglyceric acid + A.D.P. ⇅ 3-Phosphoglyceric acid + A.T.P.	Diphosphoglyceric dephosphorylase	Crystallised by Bücher (1942)
3-Phosphoglyceric acid ⇅ 2-Phosphoglyceric acid	Triosemutase (Phosphoglyceromutase)	Not purified
2-Phosphoglyceric acid ⇅ 2-Phosphoenolpyruvic acid	Enolase	Metalloprotein (magnesium, manganese or zinc). Crystallised by Warburg and Christian (1941, 1942)
2-Phosphoenolpyruvic acid + A.D.P. ⇅ Pyruvic acid + A.T.P.	Phosphopyruvate dephosphorylase	Metalloprotein (magnesium, and possibly potassium). Lardy and Ziegler (1945). Crystallised by Kubowitz and Ott (1944)

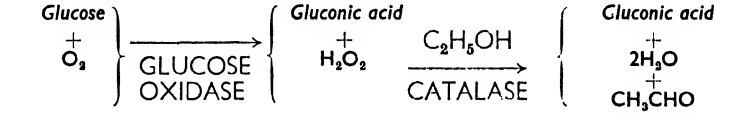
a key substance for various types of metabolism and from it arises, according to the organism and type of respiration, a multitude of intermediate metabolites.

Aerobic Carbohydrate Breakdown

Phosphorylative degradation, as described in the preceding section, is probably common to both aerobic and anaerobic cells, but an alternative phosphorylative pathway for glucose oxidation in aerobes certainly exists. Unicellular and multicellular organisms possess, as already mentioned, a pyridine-nucleotide enzyme, glucose-6-phosphate dehydrogenase, which oxidises its substrate to phosphogluconic acid. Dickens (1936, 1938) suggests that phosphogluconic acid then undergoes a series of decarboxylations and oxidations with final production of pyruvic acid. Little is known about the importance of this non-fermentative oxidation, but it should be kept in mind as an example of an alternative metabolic pathway which may play a greater or lesser part in cellular economy, according to environmental conditions. Certain micro-organisms are able to oxidise glucose, although unable to ferment it, and some of these are even unable to utilise pyruvate anaerobically (Barron and Friedemann, 1941).

Other aerobic micro-organisms are capable of direct oxidation of carbohydrate without preliminary phosphorylation, but the relative importance of such non-phosphorylative oxidation is unknown. An enzyme glucose oxidase or notatin, a flavoprotein, has been isolated from the mould *Penicillium notatum* (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw and Raistrick, 1945). The enzymes catalysing subsequent stages in non-phosphorylative oxidation are unknown and the nature of the oxidative pathway can only be surmised (Tamiya, 1942; Muller, 1929). Glucose oxidase oxidises glucose to gluconic acid, and is capable of transferring hydrogen from metabolite directly to molecular oxygen without intervention of the cytochrome system; in which case hydrogen peroxide is produced. This is characteristic of a group of enzymes known as *aero-dehydrogenases*; other members of the group, such as xanthine

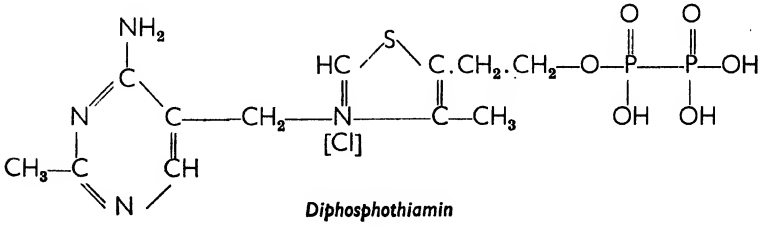
oxidase, have also been purified and identified as flavoproteins (Ball, 1939). Hydrogen peroxide is highly toxic to most living cells, and if produced by the aero-dehydrogenases must be destroyed instantaneously. Keilin and Hartree (1945) have suggested that oxidation by the aero-dehydrogenases is coupled with oxidation of other substrates, such as alcohols, through the enzyme catalase, a hæmatin-protein enzyme which occurs in all aerobic cells. The complete reaction for glucose oxidase could then be represented as follows :—



Degradation of pyruvic acid

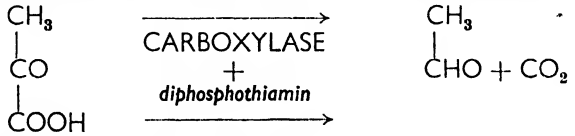
The pyruvic acid molecule is highly reactive and participates in a wide variety of biological reactions. Barron (1943) has suggested that it should be regarded as the "hub towards which converge carbohydrates, fats and proteins in their catabolic and anabolic reactions." It can probably be regarded also as the dividing point between aerobic oxidative pathways involving complete oxidation of foodstuffs to carbon dioxide and water, and the more wasteful anaerobic metabolisms involving accumulation of such metabolic fragments as lactic acid, acetic acid, alcohol, butyric acid and so on.

At present, the cellular metabolism of pyruvate is incompletely understood and the enzyme systems concerned have not been studied with the same intensity as the enzymes of glycolysis. Diphosphothiamin (*co-carboxylase*) plays an essential part in many of these enzymic reactions, but the exact way in which it participates is unknown (*cf.* Karrer and Viscontini, 1946).

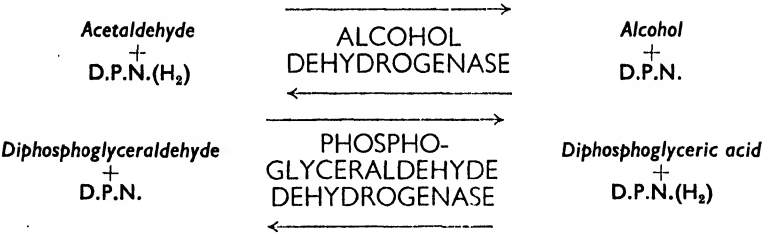


Diphosphothiamin

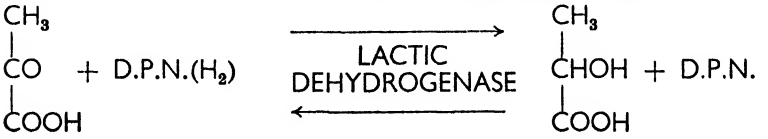
Diphosphothiamin was identified by Lohmann and Schuster (1937) as an essential coenzyme for the enzyme system in yeast which catalyses the anaerobic decarboxylation of pyruvic acid (carboxylase). Carboxylase was subsequently purified



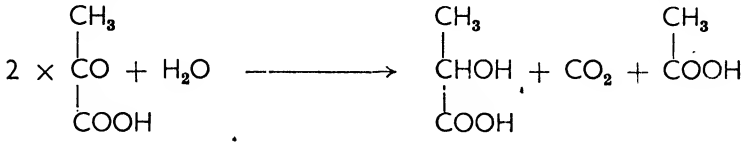
and found to require magnesium as well as co-carboxylase (Green, Herbert and Subrahmanyan, 1941). In the absence of oxygen, the acetaldehyde formed from pyruvate is reduced to alcohol by alcohol dehydrogenase, hydrogen being provided by dihydro-diphosphopyridine nucleotide derived, for example, from oxidation of diphosphoglyceraldehyde (see p. 66). As we have noted, this reaction provides a possible mechanism for the re-entry of pyridine nucleotide into the anaerobic cycle at the phosphoglyceraldehyde stage. For each molecule of phosphoglyceraldehyde oxidised to phosphoglyceric acid, one molecule of acetaldehyde may be reduced to alcohol.



Many types of cells do not produce alcohol as the main end-product of fermentation, but a number of other fermentation products can also be accounted for by the varied reactions of pyruvate metabolism. Lactic acid is produced from pyruvic acid by a reduction again involving dihydrodiphosphopyridine nucleotide, so providing another route by which the pyridine nucleotide can be regenerated. The enzyme (lactic dehydrogenase) from animal cells has been crystallised (Straub, 1940).



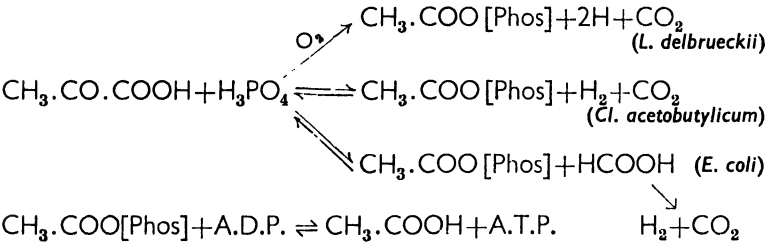
Acetic acid may arise by a dismutation reaction in which one molecule of pyruvate is oxidised, while a second is reduced to lactic acid. The enzyme catalysing this reaction has not



been purified, but diphosphothiamin seems to be involved as a coenzyme.

Pyruvate may also be decarboxylated by oxidative reactions which usually produce acetic acid. In bacteria, preliminary conjugation of pyruvate with phosphate may occur, with acetyl phosphate formed as a labile intermediate product. This type of reaction is known as *phosphoroclastic* splitting (review, Lipmann, 1946*a*). The overall reaction varies with conditions and bacterial species, but acetyl phosphate is formed in all cases; it may be converted to acetic acid, or may condense directly with other carbon compounds to give 4 or 6 carbon fragments.

Three known types of phosphoroclastic splitting of pyruvate by bacteria are compared in the following equations :—



The top equation represents the first steps in the aerobic conversion of pyruvic acid to acetic acid and carbon dioxide by *Lactobacillus delbrueckii*. The reaction only proceeds in the presence of oxygen and phosphate; acetyl phosphate has been isolated as a crystalline salt from the reaction medium by Lipmann (1944). Hydrogen is removed by some unknown hydrogen acceptor.

The other two equations depict the mechanism of anaerobic transformation of pyruvate to acetic acid, carbon dioxide and

hydrogen. *Escherichia coli* forms formic acid as an intermediate product and decomposes it by the enzyme formic hydrogenlyase; *Clostridium acetobutylicum* forms carbon dioxide and hydrogen directly. Diphosphothiamin and magnesium or manganese have been shown to be essential factors for the reaction in *E. coli*. Here again, labile acetyl phosphate has been demonstrated to be a preliminary product in both cases (Koepsell and Johnson, 1942; Kalnitsky and Werkman, 1943; Utter and Werkman, 1944).

Acetyl phosphate contains an energy-rich phosphate bond, which can be transferred to adenosine diphosphate, allowing the energy released by decarboxylation to be utilised for endergonic reactions. The process may be reversed, and pyruvate may be formed from acetate and carbon dioxide; in this case energy is supplied by adenosine triphosphate. The occurrence of such a process has been directly proved in cell-free extracts of *E. coli*, which do not contain formic hydrogenlyase. These extracts form pyruvate either from formate and acetyl phosphate or from formate, acetate and adenosine triphosphate (Lipmann and Tuttle, 1945; Utter, Lipmann and Werkman, 1945). In all decarboxylation reactions, equilibrium is in favour of carbon dioxide formation, so that rapid removal of pyruvic acid must be effected to induce the reverse reaction.

Direct oxidative decarboxylation of pyruvate without intermediate phosphorylation occurs in micro-organisms and in animal tissue (Stumpf, 1945). This reaction is strongly inhibited by fluoroacetate, which acts as a specific inhibitor of acetate oxidation and probably prevents pyruvate breakdown by causing an accumulation of acetic acid (Bartlett and Barron, 1947; Kalnitsky and Barron, 1947). Fluoroacetate had no effect on acetyl phosphate formation by *E. coli*, and only partially inhibited pyruvate oxidation by *E. coli*, gonococci and *Corynebacterium creatinovorans*. In these organisms, at any rate, pyruvate evidently need not necessarily be metabolised directly to acetate.

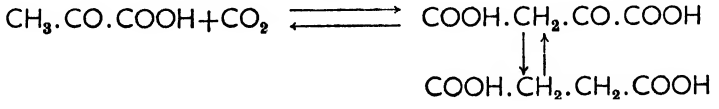
The exact course of pyruvic acid decarboxylation in animal cells is less clear than in micro-organisms. The use of fluoroacetate suggested that a large proportion of pyruvate

oxidation goes through acetate (Bartlett and Barron, 1947). The formation of acetyl phosphate has not yet been demonstrated; because of the universal occurrence of an enzyme which splits acetylphosphate with great rapidity, its participation in pyruvate metabolism cannot be easily proved (Lipmann, 1946*a*). It is, however, reasonable to assume that pyruvate breaks down in animal cells to a two-carbon compound which might be acetate or the acetyl radical (see review, Bloch, 1947).

Condensation of pyruvic acid; tricarboxylic acid cycle and cellular synthesis

The reactions of pyruvate already discussed are part of the energy-yielding mechanisms of cellular metabolism, and the energy made available by these reactions can be utilised for the synthesis of new protoplasm. Pyruvate is not only a key substance in exergonic reactions, but also performs an essential part as a building block for the formation of carbohydrates, fats and proteins. The reactions involved are by no means fully understood, and the majority of the enzymes catalysing amino acid and fat synthesis have yet to be isolated; the best that we can do is to sketch an indistinct outline of the pattern of cell synthesis.

Micro-organisms will not grow if completely deprived of carbon dioxide by a rapid stream of carbon dioxide-free air. This failure to grow is probably associated with the failure of the synthetic process of carbon dioxide fixation. The existence of such a process in non-photosynthetic organisms was indicated when Wood and Werkman (1938) showed that during fermentation of glycerol by propionic acid bacteria, a stoichiometric relationship existed between carbon dioxide utilisation and succinic acid formation. Pyruvic acid was known to be an intermediate product in this fermentation, and the suggestion was put forward that pyruvate combined with carbon dioxide to form oxaloacetic acid, which was then reduced to succinic acid. More recently an enzyme, oxaloacetic carboxylase, was



found in bacterial and liver extracts; it catalyses reversibly the decarboxylation of oxaloacetic acid (Krampitz, Wood and Werkman, 1943; Krampitz and Werkman, 1941). Thus, carbon dioxide fixation was established as a definite reversible enzymic process (Ochoa, 1946). A similar type of reaction has been identified in the carboxylation of α-ketoglutaric acid to oxalosuccinic acid by oxalosuccinic carboxylase (Ochoa, 1945).

The enzyme systems involved in these fixation reactions have not yet been purified, but they are known to require magnesium or manganese; there is some evidence that, in certain micro-organisms, biotin may be involved (Lardy, Potter and Elvehjem, 1947; Shive and Rogers, 1947). Adenosine triphosphate has also been shown to be required for full activity of an oxaloacetate decarboxylase from liver (Utter and Wood, 1946). The fixation of carbon dioxide in oxaloacetate is an endergonic reaction; adenosine triphosphate may well supply the requisite energy. Phosphorylation probably is also associated with carbon dioxide fixation when pyruvate is formed from acetic acid via acetyl phosphate, the reverse of "phosphoroclastic" splitting of pyruvate referred to on p. 75 (Lipmann and Tuttle, 1945). Like carboxylation of acetate, these carboxylation reactions have their equilibria in favour of carbon dioxide formation, and the keto acids formed must therefore be removed rapidly through the action of pyridine nucleotide dehydrogenases. The keto acids, and their dehydrogenases, play an important part in the oxidative system, known as the *tricarboxylic acid* (or Krebs) cycle.

We have still to account for a method of oxidation of acetic acid, the product of decarboxylation of pyruvate; this is believed to occur by means of the tricarboxylic acid cycle (Krebs, 1943; Wood, 1946). Oxaloacetate condenses with either acetic acid or the acetyl radical derived from decarboxylation of pyruvate (we do not yet know whether acetyl phosphate plays a role in this reaction). A series of successive enzymic reactions, involving an oxidative step, gives rise to α-ketoglutaric acid and carbon dioxide (Fig. 8).

α-Ketoglutaric acid may on further oxidation and decarboxylation through succinic, fumaric and malic acids

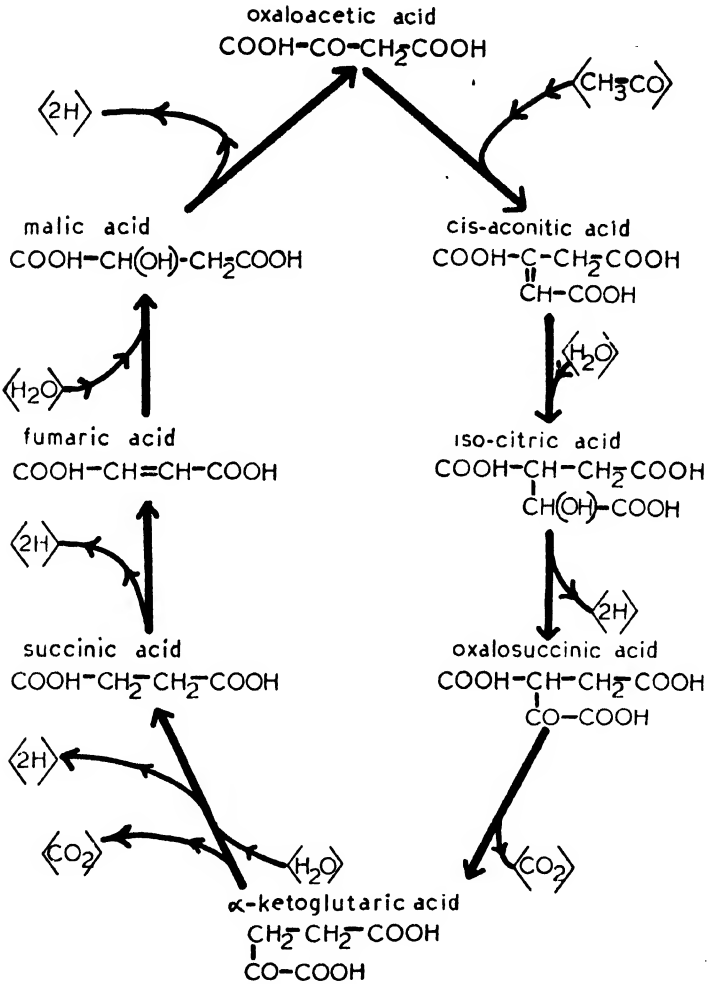


FIG. 8.—The tricarboxylic acid cycle.

regenerate oxaloacetic acid, which can then partake in another cycle. Thus we find that for each complete cycle, one molecule of acetate (derived from one of pyruvate with loss of carbon

dioxide) is completely oxidised to two molecules of carbon dioxide.

Although there is still considerable doubt as to the detailed pathways involved, there is little doubt that some such cycle exists in most cells. Evidence for the cycle originally depended largely on the following facts observed with minced muscle (Krebs, 1943). The conclusions reached can probably be extended also to micro-organisms (Speck, Moulder and Evans, 1946).

(1) All the component acids of the cycle are rapidly metabolised when added to cells under aerobic conditions.

(2) Small amounts of these acids catalyse the oxidation of pyruvate.

(3) Pyruvate oxidation is inhibited by malonate, which is a specific inhibitor for succinic dehydrogenase. In the presence of malonate, succinic acid accumulates when either pyruvate, or fumarate or malate is added. In malaria parasites, acetate is also found to accumulate under these conditions.

Enzymes are known which catalyse all the reactions of the cycle, but few of them have been obtained pure, and some of their coenzymes are still unknown (Lardy and Elvehjem, 1945). Much remains to be done before final proof of the cycle is obtained. Evidence is accumulating that here too phosphorylation is involved, while pyruvate oxidation in the malaria parasite needs diphosphothiamin, both pyridine nucleotides, adenosine triphosphate and manganous ions (Speck, Moulder and Evans, 1946). Evidence for the participation of acetate in the cycle has been provided by the action of fluoroacetate in inhibiting conversion of acetate to citrate (formed enzymically from *iso*-citrate). In tissue slices, none of the oxidative steps in the cycle (*i.e.* oxidation of iso-citric, α-ketoglutaric, succinic or malic acids) were affected by fluoroacetate (Bartlett and Barron, 1947).

The keto acids taking part in the tricarboxylic acid cycle are acted on by specific pyridine nucleotide dehydrogenases and so can be oxidised through the cytochrome system. Succinic

acid is oxidised to malic acid by succinic dehydrogenase, an enzyme which has not yet been purified and does not fit into any known group of enzymes. It acts through the cytochrome system, but cannot transfer hydrogen to cytochrome through any known pyridine nucleotides or flavoproteins; the intermediary factor is an unknown soluble heat-labile substance (Stoppani, 1947).

The tricarboxylic acid cycle may seem to be over-complicated for the oxidation of a small molecule such as acetic acid. However, the cycle establishes a link between carbohydrate and amino acid metabolism which illustrates well the close integration between degradation and synthesis in the overall metabolic processes of the cell. Every step is essentially reversible, and this reversed cycle provides a means for the reductive fixation of carbon dioxide with ultimate production of pyruvate from which a variety of metabolites may be synthesised (Fig. 9).

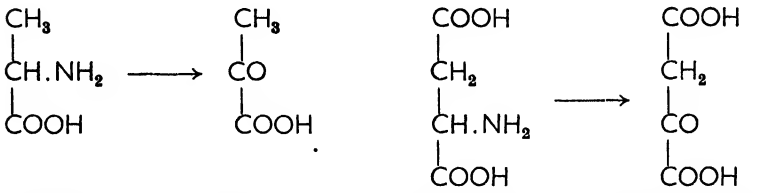
Transamination

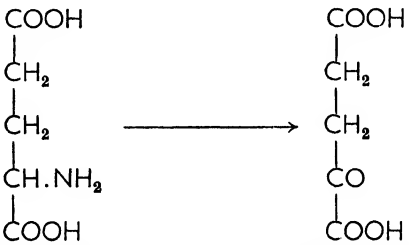
We have already mentioned the aero-dehydrogenase group of enzymes which can transfer hydrogen directly from metabolite to oxygen. The flavoprotein, *d*-amino acid oxidase, is another member of this group; it catalyses the oxidative deamination of *d*-amino acids to α-keto acids, with formation of hydrogen peroxide and ammonia (Warburg and Christian, 1938).

$$\mathrm{R{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH} \xrightarrow[\text{OXIDASE}]{\text{D-AMINO ACID}} \mathrm{R{-}\overset{\displaystyle O}{\overset{\|}{C}}{-}COOH} + H_2O_2 + NH_3$$

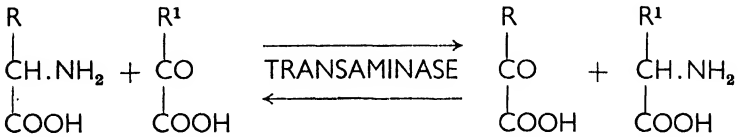
Flavoproteins are known which specifically deaminate certain natural *l*-amino acids in the same way (Blanchard, Green, Nocito and Ratner, 1945). Three of the keto acids whose formation we have traced in carbohydrate metabolism can be formed by this type of reaction. Alanine yields pyruvic acid, aspartic acid yields oxaloacetic acid, while glutamic acid yields α-ketoglutaric acid. Six other commonly occurring

amino acids are known to yield one or other of these α-keto acids indirectly on oxidation.

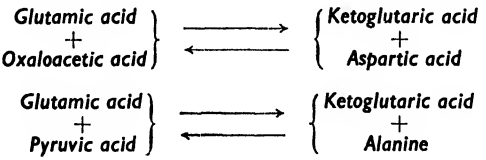




The reverse process, amination of α-keto acids, does not appear to occur directly, but has been found to take place by a transamination reaction. Enzymes known as *transaminases* catalyse the transfer of an amino group between certain α-keto acids and amino acids (Herbst, 1944).

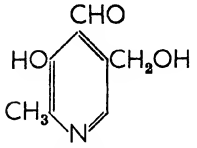


The most completely understood of known transaminations are those involving glutamic acid and oxaloacetic or pyruvic acids. There is no reason to believe that transfer mechanisms

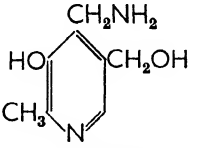


will not be found involving other simple amino acids. Transaminases have been purified to some extent, and are known

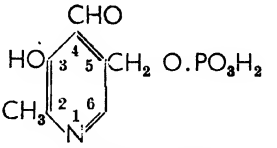
to require pyridoxal (vitamin B_6) or its phosphate for activity (Lichstein, Gunsalus and Umbreit, 1945; Umbreit, O'Kane and Gunsalus, 1946; Schlenk and Fischer, 1947; O'Kane and Gunsalus, 1947). The aldehyde radical in pyridoxal is known to be easily and reversibly exchanged for an amino group; pyridoxal may therefore act as a coenzyme for transamination by transporting amino groups.



Pyridoxal



Pyridoxamine



Pyridoxal-phosphate

There has been disagreement as to the position of the phosphate residue in pyridoxal phosphate. Pyridoxal-acetal-phosphate, in which the phosphate is attached to the phenolic hydroxyl at position 3, was found to have limited coenzyme activity in amino acid decarboxylation (p. 135) and no activity as a co-transaminase (Karrer and Viscontini, 1947 *a*, *b* and *c*). The alternative position of the phosphate on the alcoholic hydroxyl group at position 5 is more probable, since a compound of this structure had high co-decarboxylase activity and was also active as a co-transaminase (Gunsalus and Umbreit, 1947).

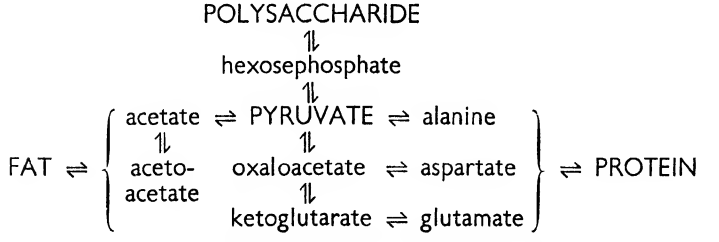


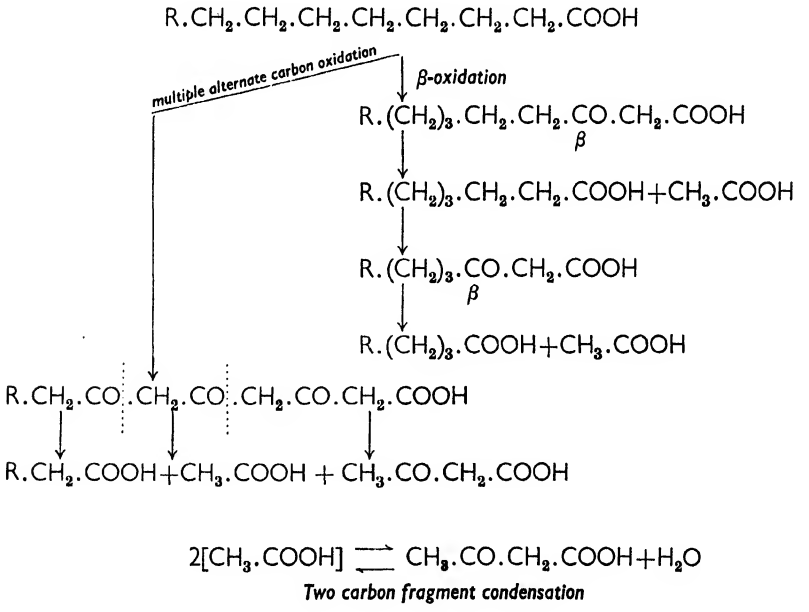
FIG. 9.—Relation between carbohydrate, amino acid and fat metabolism.

The connection between amino acid and carbohydrate metabolism now becomes obvious (Kritzman, 1947; review, Stotz, 1945). Once again pyruvate occupies a key position, and the reason for its participation in the tricarboxylic acid cycle becomes more evident. We can trace a mechanism for formation of simple amino acids from the products of

carbohydrate breakdown or carbon dioxide fixation. This mechanism may be represented diagrammatically as in Fig. 9.

Fat metabolism

Relatively little is known of the nature of fats synthesised by micro-organisms, or of the degradative pathways followed in their utilisation, but the general trend of comparative biochemistry is to find close similarities between metabolic pathways in higher forms of life and in micro-organisms. Fatty acids are believed to be metabolised in animal cells by oxidation at alternate carbon atoms, starting with the β-carbon, with formation of 2 or 4 carbon fragments; the possibility of some ω-oxidation cannot be excluded. The two carbon fragments can be acetate or acetyl phosphate; the four carbon fragments appear as acetoacetate, which can also be



formed by condensation of a pair of two carbon fragments. β-Oxidation may proceed either by a series of discrete steps, or by multiple alternate-carbon oxidation in which numerous

two or four carbon fragments are produced simultaneously (Buchanan, Sakami and Gurin, 1947 ; Breusch and Ulusoy, 1947 ; see also review by Stadie, 1945).

The use of labelled carbon has shown that fatty acid metabolism is a reversible process in which acetate, adenosine triphosphate and the tricarboxylic acid cycle all play important parts (review, Wood, 1946). The participation of adenosine triphosphate suggests that phosphorylation may occur (Lehninger, 1945). Fatty acid metabolism in the presence of malonate leads to accumulation of acetoacetate and *iso*-citrate ; as malonate inhibits one step in the tricarboxylic acid cycle, this is further evidence for participation by the cycle in fat oxidation (Breusch, 1943 ; Lehninger, 1946 *a* and *b* ; Floyd, Medes and Weinhouse, 1947).

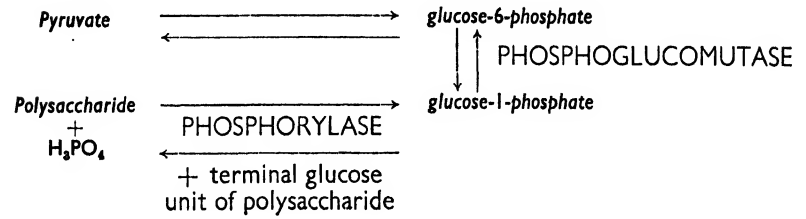
In bacteria the early results of Stephenson and Whetham (1922) indicated that the amount of lipoid synthesised by the Timothy grass bacillus was influenced by the composition of the medium and was greatly increased in the presence of acetate. The conversion during bacterial fermentation of " labelled " acetate containing isotopic carbon to butyric and caproic acids has been demonstrated (Wood, Brown and Werkman, 1945 ; Barker, Kamen and Bornstein, 1945). The enzymes of all these conversions have yet to be purified, but there seems good reason to believe that in micro-organisms the tricarboxylic acid cycle provides a mechanism, not only for the oxidation of acetate, but also for the integration of carbohydrate, fat and amino acid metabolism.

Carbohydrate synthesis

Carbohydrate breakdown by the living cell is a metabolic process which is to some extent understood. Much remains to be elucidated, particularly with regard to aerobic respiration, but the sequence of enzyme-catalysed reactions from the phosphorylation of hexose to the production of pyruvic acid has been fully verified and even largely reconstructed with pure enzymes under artificial conditions. Such experiments, however, only show that postulated reactions are possible ; they cannot show their relative importance or reproduce the

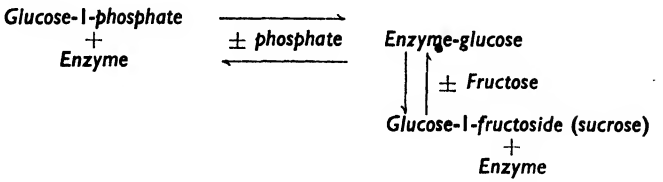
directive influences under which they operate in the living cell. All the steps in the anaerobic breakdown of carbohydrate to pyruvate are reversible, so that the same enzyme systems may either degrade or synthesise a particular substrate according to local cellular conditions (Lardy and Ziegler, 1945).

The aim of synthetic processes is either to form essential cell structures and enzymes, or to store surplus metabolites for future use. The water-solubility of glucose and the high osmotic pressure of its solutions makes it unsuitable as a form of carbohydrate store; therefore, reversal of the phosphorylative breakdown of glucose or any of its intermediates often leads to formation of polysaccharides. During the synthetic process, glucose-6-phosphate is converted by a metalloprotein enzyme, phosphoglucomutase, to glucose-1-phosphate which under the influence of a phosphorylase polymerises to a polysaccharide, the nature of which depends upon the particular phosphorylase and on the nature of the polysaccharide already present in the cell. A crystalline phosphorylase has been isolated from muscle by Green and Cori (1943), and its action has been shown to be the exchange of the phosphate radical of glucose-1-phosphate for the terminal glucose unit of the polysaccharide already present. The scheme for carbohydrate synthesis from pyruvate may be represented as follows :—

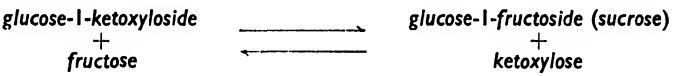


The synthesis of sucrose by *Pseudomonas saccharophila* has been shown to occur through the action of the enzyme sucrose phosphorylase (Hassid, Doudoroff and Barker, 1947; Doudoroff, Barker and Hassid, 1947; Doudoroff, Hassid and Barker, 1947). The primary reaction consists of a combination of glucose phosphate with the enzyme, followed

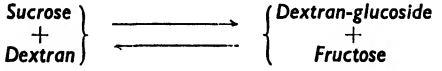
by liberation of phosphate, after which glucose combines with fructose :—



The overall reaction is in effect a transfer of glucose, and since it is reversible, the acceptor may be either a phosphate or a fructose radical. The transfer reaction may, in fact, proceed in the absence of a phosphorylated intermediate with an extraordinary variety of acceptors other than phosphate. For example, many ketose sugars and even certain aldoses will accept glucose, with the result that interconversion of disaccharides may be effected, as may be illustrated by the production of sucrose from glucosidoketoxyloside.



The synthesis or degradation of dextran (a glucosan) is accomplished by an enzyme from *Leuconostoc mesenteroides* without addition of phosphate, and no phosphorylated intermediates can be isolated (Cori *et al.*, 1945). The reaction probably involves exchange of one glucosidic linkage for another. The dextran produced *in vitro* by the isolated



enzyme was identical immunologically with that found in the living organism (Hehre, 1943). There is evidence that levans are produced by a similar mechanism.

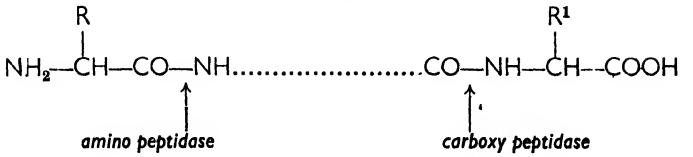
Protein synthesis

Our knowledge of carbohydrate synthesis is based on the reversibility of the enzyme systems taking part in carbohydrate breakdown. Unfortunately, the same cannot be said about

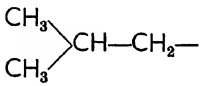
protein synthesis, about which we know practically nothing. Proteins possess a high degree of specificity, each species building its own characteristic pattern ; even in the same cell, the number of different proteins must be large to account for the multifarious enzymes present. Enzymes are in addition species specific, as is shown by the immunological properties of catalase and trypsins from different species (Campbell and Fourt, 1939 ; Northrop, 1939). Different coenzymes are often required by the same enzyme isolated from different organisms ; thus, enzymes concerned with pyruvic acid utilisation appear to require either magnesium or manganese as one of their co-factors when isolated from bacteria, but use manganese if they are of animal origin. There is some evidence that the pyridine nucleotide required by malic and glutamic dehydrogenases varies with the source of enzyme (Schlenk, 1945), but this cannot be confirmed until the enzymes in question are purified. The *p*H for maximum activity of enzymes also varies with the source.

This high degree of specificity among individual proteins points to a similar specificity in the enzyme systems responsible for their synthesis. At present, due to lack of knowledge and to the complexity of the problem, our investigations on specificity are limited to the action of the numerous proteolytic enzymes which degrade proteins and polypeptides. These enzymes, which all hydrolyse the peptide bond, may be divided into two main groups, according to their site of attack (Bergmann, 1942). One group acts only on terminal peptide bonds and can be classed as " exopeptidases," while the second group, the " endopeptidases," are capable of attacking centrally-located peptide bonds and, to a lesser extent, the terminal bonds as well. The older terminology, still in use, refers to these groups as peptidases and proteinases respectively. Within these groups, there are considerable differences in specificity between individual enzymes ; the hydrolysis of a given peptide link by a given enzyme appears to be determined largely by the nature of adjacent and nearby amino acids and of the end groups. For example, exopeptidases are subdivided into aminopeptidases and carboxypeptidases, the

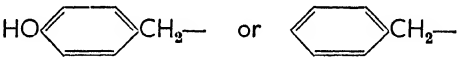
peptide group which is split being adjacent to either a free amino group or a free carboxy group respectively.



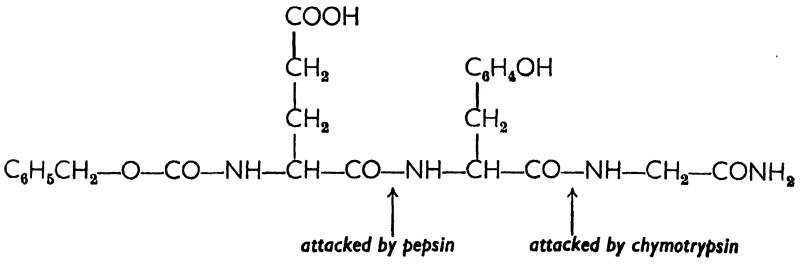
As well as showing this broad specificity, the individual peptidases also require certain groups in the side chain attached to the carbon atom adjacent to the peptide bond. For example, certain amino peptidases will only split a bond in which R is represented by



i.e. the terminal amino acid is leucine, while some carboxy peptidases are known for which R must be



that is tyrosine or phenylalanine are the terminal amino acids split. In the same way, the endopeptidases require certain side groups adjacent to the peptide link, and the specific amino acid thus involved is attacked either through its carboxy or its amino group. This is well illustrated by the action on the same synthetic substrate (carbobenzoxy-*l*-glutamyl-*l*-tyrosyl-glycineamide) of pepsin and chymotrypsin, both of which attack a peptide link adjacent to an aromatic amino acid (Bergmann and Fruton, 1941).



A considerable degree of optical specificity is shown by some proteases and peptidases, so that peptides containing *d*-amino acids in certain positions are not hydrolysed (Bergmann and Fruton, 1941 ; Stahmann, Fruton and Bergmann, 1946). On the other hand, peptidases are known which hydrolyse peptides containing *d*- as well as *l*-amino acids. Usually the *l*- form is hydrolysed faster than the *d*- form, but some bacterial peptidase preparations split the two forms at approximately equal rates (Berger, Johnson and Baumann, 1941). There is evidence that hydrolysis of *d*-amino acid peptides may be due to an enzyme different from that hydrolysing the natural isomers (Maschmann, 1942, 1943) ; proof must await actual separation of both enzymes. An alternative suggestion has been made that the same enzyme is involved, but that optical specificity is changed by the presence of certain metals or reducing agents (Bayerle and Borger, 1942 ; Bayerle and Reiffert, 1942).

The part played by proteolytic enzymes in protein synthesis is largely conjectural. We are not yet able to assess to what extent, if any, the reversal of proteolytic hydrolysis is responsible for the synthesis of peptide bonds. As Bergmann and Fruton (1944) point out, " perhaps the strongest reason for believing that the enzyme-catalysed condensation of amino acids represents the most probable metabolic course of protein synthesis is the fact that the proteolytic enzymes, by virtue of their sharp specificity, are the only known biocatalysts which could direct, precisely and reproducibly, the complex sequence of successive peptide syntheses required for the formation of a protein."

The enzymic hydrolysis of a peptide bond is, like other enzymic processes, an equilibrium process, but equilibrium is overwhelmingly in favour of hydrolysis. Therefore, the synthesis of a peptide can only occur when the product of reaction is rapidly removed, as is possible in the cell where coupled reaction sequences occur. The *in-vitro* enzymic synthesis of peptides has been achieved by Behrens and Bergmann (1939), using derivatives of amino acids which gave insoluble peptides. For example, the action of papain on a mixture of

benzoyl-leucine and leucine-anilide formed benzoyl-leucyl-leucine-anilide, which is so insoluble in water that a saturated solution is always at a lower concentration than the equilibrium concentration. As a result, the dipeptide was continually removed from solution by crystallisation and the reaction proceeded in the direction of synthesis. Other examples of similar syntheses achieved by Bergmann are as follows :—

Benzoyl-phenylalanine + leucine-anilide	PAPAIN →	*benzoyl-phenylalanyl-leucine-anilide*
Carbobenzoxy-phenylalanyl-glycine + tyrosine-amide	PAPAIN →	*carbobenzoxy-phenylalanyl-glycyl-tyrosine-amide*
Benzoyl-tyrosine + leucine-anilide	CHYMOTRYPSIN →	*benzoyl-tyrosyl-leucine-anilide*
Benzoyl-tyrosine + glycine-anilide	CHYMOTRYPSIN →	*benzoyl-tyrosyl-glycine-anilide*

The synthesis of a peptide bond requires the provision of a considerable amount of energy (about 3 kg. cals/mol.). From our knowledge of such reactions, we may postulate that this energy may be provided by coupled reactions. A synthesis of a polypeptide coupled with hydrolysis of another peptide has been demonstrated *in vitro* by Bergmann through the action of the enzyme papain on the peptides acetyl-*dl*-phenyl-alanyl-glycine and glycyl-*l*-leucine. Although neither of the peptides is hydrolysed by the enzyme, an equilibrium is soon set up in solution, with formation of a very small amount of acetyl-*dl*-phenylalanyl-glycyl-glycyl-leucine. This tetrapeptide is at once hydrolysed in two successive steps in which first leucine, and then glycine, is split. The equilibrium is thus upset and further tetrapeptide is synthesised and in turn hydrolysed until all the glycyl-leucine is used up by the synthetic reaction.

Acetyl-phenylalanyl-glycine + glycyl-leucine

↓ ↑

Acetyl-phenylalanyl-glycyl-glycyl-leucine

↓ ↑

Acetyl-phenylalanyl-glycyl-glycine + leucine

↓ ↑

Acetyl-phenylalanyl-glycine + glycine + leucine

The nett result is hydrolysis of glycyl-leucine without change of the acetyl peptide. The existence of such a type of reaction

suggests that in the cell, peptide synthesis and breakdown may be interconnected processes, influenced by the presence of other peptides or proteins.

Speculation enables us to envisage many other coupled reactions which may provide energy for peptide synthesis. Energy transfer might occur through phosphorylation, as in carbohydrate metabolism; the carboxyl or amino group of amino acids, if phosphorylated, would be energy-rich, and could thus couple with another amino acid with liberation of free phosphate. Energy may also be transferred from oxido-reduction systems in other ways as yet unknown. The possible relation of peptide synthesis to energy supplied through the phosphate bond is suggested by the discovery of two enzymes which catalyse formation of the —CONH— bond, both of which require adenosine triphosphate and magnesium. One enzyme from rat liver and kidney synthesises a peptide bond between *p*-aminobenzoic acid and glycine (Cohen and McGilvery, 1947); the other, found in sheep brain and *Staph. aureus*, catalyses the formation of glutamine from glutamic acid (Elliott, 1948; Elliott and Gale, 1948).

An alternative method of peptide synthesis has been suggested, namely coupling of a keto acid, *e.g.* pyruvic acid, with one or two molecules of an amino acid amide with intermediate formation of dehydroamino acid peptides or di(acylamino)-propionic acid peptides (Bergmann and Fruton, 1944; Gonçalves and Greenstein, 1948).

The synthetic route for formation of the nucleic acid moiety of nucleoproteins is also largely unknown. There is evidence that the first step, nucleoside formation, involves a phosphate exchange as in polysaccharide synthesis, since the synthesis is known to occur by the action of a nucleosidase enzyme on ribose-1-phosphate and purine (Cori, Hassid, Doudoroff and Kalckar, 1945; Kalckar, 1945, 1946, 1947). In view of this phosphorylative step, Kalckar suggested that the enzyme be known as a nucleosidase phosphorylase.

Conclusion

It should be evident from this brief outline that the whole process of cellular metabolism is a closely woven, interlocking and interdependent series of enzymic reactions. Disturbance of any one enzymic reaction by introduction of a poison or drug will extend far beyond that single reaction ; if the cell survives, there will occur an overall adjustment in enzymic balance which may become evident either in some fermentative change, in altered demand for foodstuff, in a changed rate of growth or in altered response to further contact with drugs.

Since whole groups of enzymes possess certain common characters, such as a common prosthetic group or coenzyme, it is probable that a drug will affect not a single enzyme but a whole group of related enzymes, some more, others less, essential to life. The problem of reducing drug action to interference with any single enzyme is therefore immense, and the study of intermediary metabolism is likely for the present to do more towards suggesting new types of drugs than towards solving the problem of the mode of action of known drugs.

CHAPTER III

ESSENTIAL METABOLITES

DURING growth, catabolism and anabolism proceed simultaneously, and the energy derived from enzyme-catalysed exergonic reactions is utilised in the production of new cellular material. New nucleoproteins, structural proteins, lipoproteins and carbohydrates are formed, new enzymes are elaborated and new prosthetic groups and coenzymes are synthesised for these enzymes. We have seen that some intermediates for synthesis are derived from the products of catabolic reactions; others must be synthesised by specialised routes or obtained preformed from the surrounding environment.

The *autotrophic* group of bacteria are capable of complete synthesis of all cellular materials from carbon dioxide and inorganic salts. Pathogenic organisms are *heterotrophic*, that is, they require a source of carbon more complex than carbon dioxide. The nitrogen requirements of pathogens vary from the ability to grow on media containing inorganic nitrogen to a complete dependence on preformed amino acids, coenzymes, purines, etc. From the viewpoint of comparative biochemistry, pathogens may be regarded as evolutionary variants which have hit on an easy method of living, obtaining some, at any rate, of their essential organic constituents by leading a parasitic existence and stealing from their hosts. As pointed out by Knight (1945), no clear distinction can be drawn between metabolites "acquired from the environment" and those "synthesised by the cell", since an organism may be able to synthesise an essential cell constituent only very slowly, and during rapid growth must depend on an external source of supply. "Hence a given substance, required as a component of one of the essential metabolic processes might appear in three different roles as a component of the nutrients. It might appear (1) as an 'essential' nutrient when its rate

of synthesis by the cell was so slow as to be insignificant; (2) as a growth stimulant, when its rate of synthesis was somewhat faster but still slow enough to be a limiting factor; or (3) as a substance not required at all for nutrition because the cell could synthesise it so fast that it was not a limiting factor in growth."

Let us consider a specific example. Certain strains of *Escherichia coli* can grow on a medium containing only inorganic salts, ammonia and glucose, and must therefore be capable of synthesising all the nitrogenous constituents of the cell. Some strains of *Eberthella typhosa* cannot grow in this medium unless tryptophan is added. The proteins of both organisms contain tryptophan, and we can conclude that *E. coli* synthesises tryptophan, whereas *Eb. typhosa* during its parasitic existence has found it preferable to derive its tryptophan from the host tissues. Tryptophan occurs as an essential intermediate in the metabolism of both organisms, but in culture media it acts as a growth factor for only one of them.

Before proceeding further, it is as well to point out the great variability of the nutritive requirements of a given species of micro-organism. It should be remembered that "the nutritive requirements for a single strain do not necessarily represent those of the species as a whole" (Snell, 1946).

We have already mentioned that the metabolic reactions of heterotrophic micro-organisms do not differ fundamentally from those of animals. Similarity does not imply identity; the usual amino acids occur in bacterial protein, but for any one species the protein is of characteristic and constant composition (Stokes and Gunness, 1946 *a* and *b*; Freeland and Gale, 1947). However, owing to the limited data available, it is by no means certain that bacterial protein is made up solely of the amino acids known to occur in animal protein (Blass and Macheboeuf, 1946). As might be expected, the nutritional requirements of micro-organisms resemble in many respects those of higher forms of life. These requirements are simply a reflection of the overall metabolism of the cell and of the

degree to which parasitism has resulted in reduction of the ability to synthesise essential intermediate metabolites. Animals may be equally well regarded as a form of life ultimately parasitic on the photosynthetic plants, and dependent on these plants for a supply of certain metabolites.

We do not propose to review here all aspects of the subject of bacterial nutrition or to attempt to list the nutritive requirements of bacteria, but rather to indicate firstly, the relationship of nutritive requirements to the enzymic make-up of the cell; secondly, the relation of nutritive requirements to the development of a parasitic form of life; and thirdly, the implications of the nutritional and metabolic viewpoint in the design of chemotherapeutic drugs. It will not be desirable to consider only pathogenic organisms, since in many cases comparison is instructive, while the underlying similarity of intracellular metabolism in pathogen, non-pathogen and host constitutes a major problem in the development of scientific chemotherapy.

Since many diseases are caused by protozoal organisms, it is evident that the subject of protozoal nutrition is as important as that of bacterial nutrition. It will not be dealt with separately here, but where a factor is known to be required by protozoa as well as by bacteria, mention will be made in the text. Many pathogenic protozoa require extremely complex media for cultivation, and some even resist culture outside the host. For example, *Entamœba histolytica* was even thought to require the presence in the medium of living bacteria, but has now been cultivated in the presence of penicillin-killed bacteria (Jacobs, 1947).

Bacterial growth

The growth of a bacterial population in a suitable medium tends to follow a fairly characteristic pattern which can be conveniently expressed in graphic form by plotting the logarithm of the number of bacteria against time. A typical curve is shown in Fig. 10.

The growth curve is arbitrarily divided into four phases.

(1) *The lag phase* (a to b).—In the early part of this phase there may be no apparent growth, or even a slight diminution in numbers, but within a short time growth becomes apparent and gradually increases in pace until the beginning of the next phase.

(2) *The logarithmic phase* (b to c).—During this phase, cell division proceeds at a constant and maximal rate. Theoretically, each cell completes a cycle of reproduction with uniform periodicity and doubles itself in unit time. It follows that

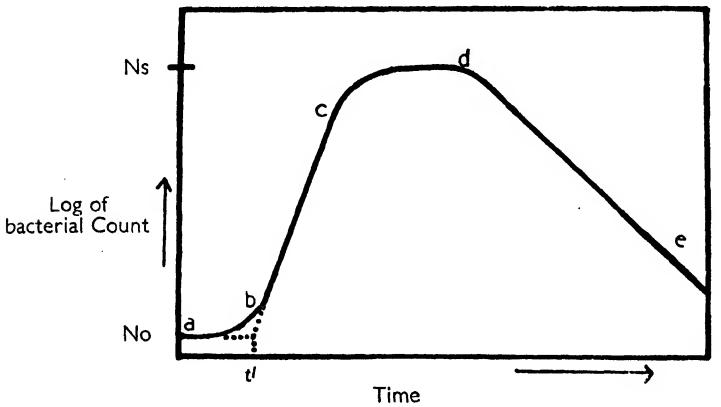


FIG. 10.—Typical bacterial population curve.

successive cell counts plotted logarithmically against time fall on an ascending straight line.

(3) *The stationary phase* (c to d).—Cells gradually cease to multiply at maximal rate until population gain from new division is more or less balanced by death of a similar number of cells.

(4) *Phase of decline* (d to e).—The stationary phase is followed by a period of steady decrease in the number of living organisms, during which reproduction ceases and, after a long time, the culture becomes sterile.

The course of growth is usually characterised by three constants, the length of the lag phase (t^l), determined by extrapolation of the logarithmic growth curve back to the

value $n = N_0$; the mean generation time or the time required (during logarithmic growth) for the population count to double, and finally the maximal count (N_s), attained during the stationary phase.

The lag phase (*cf.* Hinshelwood, 1944; Winslow and Walker, 1939). The length, t^l, of the lag phase depends on a number of factors; if the cells of the inoculum are very young, t^l may be considerable, but as the age of the inoculum increases, t^l may fall nearly to zero and then increase again. The stationary period of the lag phase is essentially one of adjustment during which the inoculated cells adapt themselves to new conditions and build up in cell and medium the necessary ingredients for growth. This is well illustrated by the case of *Bacterium lactis aerogenes* which grows on a synthetic medium with ammonium salts as sole source of nitrogen. Young inoculæ show a considerable lag period when transferred to a fresh medium; but the lag can be completely removed by addition to the basal medium of a sterile culture-filtrate from a fully-grown culture (Lodge and Hinshelwood, 1943). With older inoculæ the lag depends upon the size of the inoculum. These results suggest that for growth *B. lactis aerogenes* requires some diffusible factors which during the lag phase are built up to the necessary threshold concentration. The nature of the growth factors in this case is suggested by the observation that in the presence of amino acids as sources of nitrogen, t^l is no longer affected by inoculum size or by extracts of older cultures.

In general, unfavourable media tend to lengthen the lag phase; thus with *B. lactis aerogenes* on a synthetic medium containing glucose and phosphate buffer, t^l can be increased indefinitely as the concentration of magnesium ions is reduced (Lodge and Hinshelwood, 1939). Growth can be influenced also by insufficient concentrations of carbon dioxide (Walker, 1932; Gladstone, Fildes and Richardson, 1935; Gale, 1945). Aeration of an otherwise suitable medium with carbon dioxide-free air delays growth indefinitely, but reproduction begins immediately carbon dioxide is admitted to the air stream.

As the age of the inoculum increases, t^l falls at first, and

then increases to a constant value when it cannot be eliminated by addition of culture filtrates or by change in medium. This is probably due to a decline in the activity of cell enzymes with age (Gale, 1940; Woods and Trim, 1942). Stephenson (1939) suggests that lag may result from the following causes :—

(1) Decreased permeability due to the inoculant having remained for some hours in an exhausted medium.
(2) Decreased enzyme activity due to the same cause.
(3) Time required for formation of adaptive enzymes.
(4) Time required for production of highly-reactive molecules in optimum concentration.

The first three causes cannot be eliminated by improvement of the medium and would be most marked in cells coming from old cultures.

The logarithmic phase.—Towards the end of the lag phase, there is an increase in metabolic activity, often associated with an increase in cell size, and followed very soon by cell division which accelerates to a steady maximal rate. During the early part of the logarithmic phase, cells may show a lowered resistance to bactericidal agents, but towards the end of the phase resistance increases again. During the late lag phase and at the beginning of logarithmic growth, cells adapt themselves more rapidly to changes of media than at any other time.

The stationary phase.—During growth in a limited medium, conditions gradually become less favourable due to exhaustion of metabolites, change in *p*H and accumulation of toxic products (Morel, 1941). The effects of these adverse conditions usually increase from negligibility to vital importance in a short period, and so cause a rapid transition from logarithmic to stationary phase. When the basal medium is deficient, and concentration of any one metabolite is the limiting factor on growth, the maximal cell count becomes directly proportional to the initial concentration of that metabolite in the medium. This enables the concentration of the metabolite to be determined from growth response, growth being measured

by some standard method after sufficient time for the stationary phase to be reached. Fig. 11 represents a typical growth-response curve; it shows the growth (estimated as turbidity) of a strain of *Lactobacillus arabinosus* with varying amounts of tryptophan in an otherwise complete medium (Wright and Skeggs, 1945). Fig. 13 shows normal growth curves for varying amounts of metabolite.

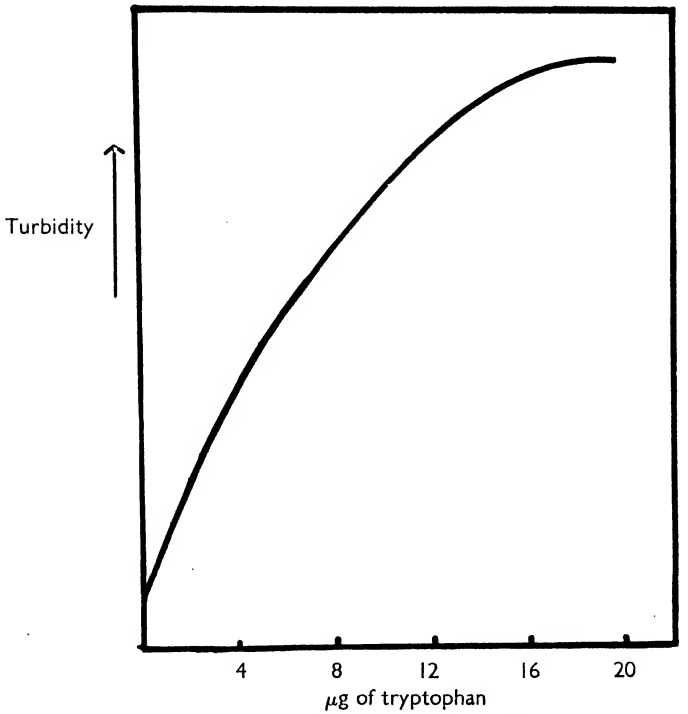


FIG. 11.—Growth response of *Lactobacillus arabinosus* to addition of tryptophan to a deficient medium after 24 hours growth. (Wright and Skeggs, 1945.)

Amino acids as essential metabolites

As noted already, some organisms such as *Escherichia coli*, *Pseudomonas pyocyanea* and Friedländer's bacillus (*Kl. pneumoniæ*) can utilise ammonia as sole source of nitrogen; they are thus able to synthesise all their amino acids. Bacteria with more exacting nutritional requirements have lost the capacity to synthesise certain amino acids; but this loss is

often incomplete, so that an organism may be " trained " to dispense with an amino acid by repeated subculture in a deficient medium. The synthetic mechanisms of exacting organisms can be regarded as having become atrophied or lost through disuse during parasitic life, when the organism could depend on the surrounding host tissue for a supply of amino acids. An organism may sometimes show a gain in growth rate in a rich medium consequent upon loss of synthetic ability (Ryan, 1946 ; Monod, 1946). In this way a pathogen may find it biologically advantageous to depend upon its host for certain essential metabolites. Such a parasitic strain is termed a " *nutritionally exacting* " strain. Certain nutritionally exacting strains of *Eberthella typhosa* grew on a simple glucose ammonium salt medium only if a small amount of tryptophan was present (Fildes, Gladstone and Knight, 1933). By serial subculture and gradual reduction in the tryptophan content of the medium, these strains became capable of growth with ammonia as sole source of nitrogen. The trained organisms did not dispense altogether with tryptophan as a constituent of their proteins but had developed the capacity to synthesise the amino acid, since chemical tests showed that tryptophan was present in non-exacting as well as in exacting strains. We do not wish to imply by the term *nutritional training* that slow reduction in tryptophan content of the medium necessarily induced a similar change in all cells of the population examined. When the nutritional requirements of a strain are determined, it is the character of a population rather than of an individual which is being measured. The character of a population may be altered either by selection of individuals from an initially heterogeneous population, or by selection of variants occurring spontaneously during growth of the population, or by induction of variation in some or all of the cells under examination. The question of the ultimate nature of bacterial variation is discussed more fully in Chapter VI.

Although tryptophan was the only amino acid found to be absolutely essential for the growth of certain exacting strains of *Eberthella typhosa*, other amino acids, namely cystine,

leucine, lysine, tyrosine and histidine had growth-promoting effects when added to a basal medium already containing tryptophan. This type of result, which is common to many studies of the nutritive requirements of micro-organisms, can be taken to indicate that although the exacting strain is capable of synthesising these other amino acids, it cannot carry out the synthesis sufficiently rapidly to meet all its requirements for maximal growth. When one growth factor is supplied, the need for another may become apparent simply because the potential rate of growth is now much greater. The limitation imposed by failure to synthesise one metabolite sufficiently rapidly is overcome by drawing on the medium for a supply of that metabolite, the whole metabolic rate is transformed to a higher plane, and the cellular requirements for every metabolite are stepped up so that another enzymic reaction becomes the limiting factor in the synthesis of new cellular material.

Some exacting strains of *Eberthella typhosa* show absolute growth requirements for amino acids other than tryptophan, such as arginine, glutamic acid or lysine. The amino acid requirements of a given strain can vary with the form of carbohydrate present in the medium (Burrows, 1942). The adaptive capacity of pathogens may be so great that, when first isolated from the host, their amino acid requirements are very much more complex than after one or two subcultures in simple media (McIlwain, Fildes, Gladstone and Knight, 1939). The capacity for adaptation may even be such that it is difficult to estimate the growth requirements of pathogenic organisms as they exist in the host. A chemotherapeutic drug may act by interfering with the utilisation of a metabolite, but if a pathogen is capable of adapting itself to dispense with that metabolite, or to synthesise sufficient for growth even in the presence of drug, then resistance to the drug may develop so rapidly as to make the drug useless as a chemotherapeutic remedy.

The amino acid requirements of *Staph. aureus* have been studied by Gladstone (1937). Strains were found to differ in their requirements and were easily trained to a less exacting

form. Some of the more exacting strains required tryptophan, cystine, leucine, valine, proline, glycine, aspartic acid, phenylalanine, arginine, histidine and methionine for full growth. Training to maximum growth in the absence of any one or all of these amino acids, with the exception of cystine, was possible, but the ease of adaptation varied both with the strain and with the amino acid. Training of a strain to become independent of one amino acid often resulted in a simultaneous reduction in its dependence on other amino acids; in other cases training to dispense with an amino acid, for example leucine, could only be accomplished readily if other amino acids were present. These results suggest a certain interdependence between the enzymic mechanisms concerned with amino acid synthesis in the cell. Streptococci have even more complex amino acid requirements than *Staph. aureus*, but here again there exist in the laboratory more and less exacting strains, and a certain amount of training is possible. By omitting each of nineteen amino acids in turn from the growth medium, Woolley (1941) was able to show that for a strain of *Strep. hæmolyticus*, glutamic acid and tryptophan were absolutely indispensable, but both together were unable to support growth as the only source of organic nitrogen. Before growth would take place, all the following amino acids had also to be present: isoleucine, lysine, arginine, cystine and tyrosine. A full account of the amino acid requirements of various lactic acid bacteria used in quantitative assay of amino acids has been given by Dunn, Shankman, Camien and Block (1947).

The very rapid change which can take place in the nutritional requirements of a pathogenic organism is well illustrated by a study of the glutamine requirements of the "Richards" strain of *Strep. hæmolyticus* (McIlwain, Fildes, Gladstone and Knight, 1939; Fildes and Gladstone, 1939). The test organism had to be maintained by repeated mouse-passage, since, after a few sub-cultures on agar, the organism became independent of added glutamine. With the untrained organism from fresh blood, a glutamine concentration of $2{\cdot}5\times10^{-6}$ M. was sufficient to allow full growth; with

smaller concentrations no growth occurred and with larger concentrations there was no augmentation of the growth rate. This " all-or-none " effect is unusual, for the response to addition of a growth factor is usually, within limits, proportional to the amount of factor added. With the exacting " Richards " strain of *Strep. hæmolyticus*, evidently preformed glutamine is essential only for the initiation of multiplication; once growth is started by a minimal amount of glutamine, more glutamine can be synthesised from other constituents of the medium. Other organisms, such as

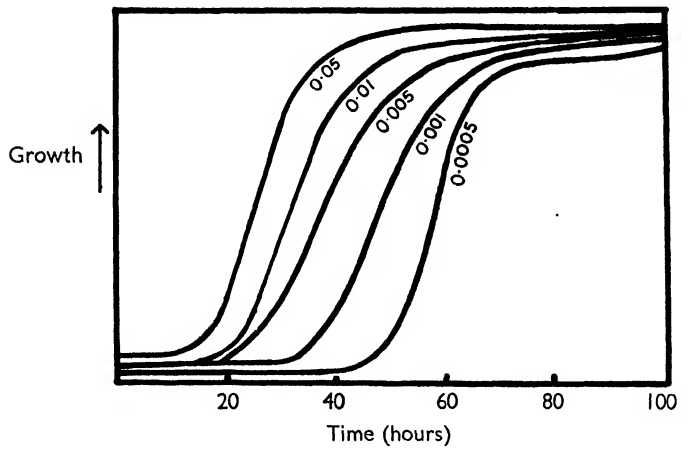


FIG. 12.—Effect of varied tryptophan concentration on the growth of typhoid bacillus in glucose-salt medium. Figures on curves represent percentage tryptophan in medium. (Burrows, 1939*a*.)

Lactobacillus casei, respond quite differently to glutamine, their growth rate showing proportionality to the amount of glutamine added to the medium.

A somewhat similar " all-or-none " response to tryptophan has been obtained with the typhoid bacillus (Burrows, 1939 *a*, *b*). Provided a minimum amount of tryptophan is added to the medium (about 0·0005 per cent.), increasing amounts of tryptophan have no effect on the growth rate as judged by the slope of the growth curve or on the total population attained, but do reduce the time lag before growth begins (see Fig. 12; also Fig. 13 where a normal growth response

to an essential metabolite is shown for comparison). When grown on a medium containing the minimal concentration of tryptophan, the organism synthesises more tryptophan and the function of the tryptophan initially added would seem to be similar to that of glutamine in the "Richards" streptococcus.

This shortening of the growth lag by tryptophan should be compared with a similar reduction in lag obtained by Lodge and Hinshelwood (1943) by addition of a cell-free

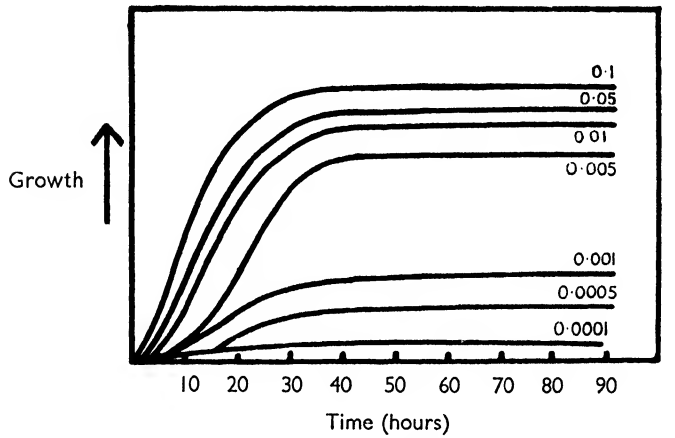


FIG. 13.—Effect of varied ammonium chloride concentration on growth of typhoid bacillus in glucose-salt medium. Figures on curves represent percentage NH_4Cl in medium. (Burrows, 1939*a*.)

filtrate of an old culture to a young culture which normally had a long lag period.

An investigation has been carried out on the synthesis of tryptophan by a strain of *Lactobacillus arabinosus* which had been trained to grow in the absence of this amino acid (Wright and Skeggs, 1945). The strain responded to the presence of tryptophan in the medium in a curious manner. The amount of tryptophan synthesised was inversely proportional to the amount present in the medium. Possibly the synthesis of tryptophan depends on an adaptive enzyme (see Chapter VI) which is only produced in direct proportion to the immediate tryptophan demand, or, alternatively, tryptophan added to

the medium may inhibit intracellular tryptophan synthesis by a mass-action effect.

These few cases should suffice to indicate the variability of microbial response to growth factors and to exemplify the underlying biochemical similarity in the amino acid requirements of bacterium and animal. Rose (1938) has listed twenty-one amino acids which are normal constituents of dietary proteins. All these can be utilised by the rat, but only ten are essential. Of the ten essential amino acids (see Table 3), nine are absolutely essential; the tenth, arginine, can be synthesised by the rat, but not at a sufficiently rapid rate to meet the needs for normal growth.

TABLE 3

Amino acids in the nutrition of the rat (Rose, 1938)

Essential Amino Acids	Non-Essential Amino Acids
Lysine	Glycine
Tryptophan	Alanine
Histidine	Serine
Phenylalanine	Aspartic acid
Leucine	Glutamic acid
Isoleucine	Proline
Threonine	Hydroxyproline
Methionine *	Citrulline
Valine	Tyrosine
(Arginine)	Cystine
	Asparagine

* Can be replaced by mixture of homocystine and choline.

The majority of these twenty-one amino acids have been shown to have growth-promoting properties for some type of micro-organism (Porter, 1946). The amino acid requirements of micro-organisms are not invariably satisfied by a mixture of all the known free amino acids. A low molecular weight polypeptide, known as strepogenin, which can be liberated from proteins, has been found to have growth-promoting properties for bacteria in an otherwise complete synthetic medium (Sprince and Woolley, 1944, 1945). Strepogenin may be related to the peptide seryl-glycyl-glutamic acid which showed limited strepogenin activity (Woolley, 1946*b*). A heat-stable factor associated with certain proteins is also

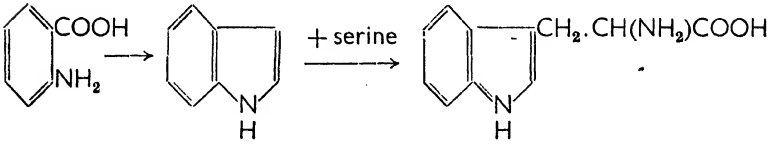
said to be required by *Lactobacillus casei* (Scott, Norris and Heuser, 1946, 1947).

Where stimulation of growth by an amino acid can be shown, the assumption is reasonable that the added amino acid plays an important part in metabolism of the organism in question. The non-essential amino acids, when present in the medium, are often deaminated or decarboxylated and then used as a source of energy and nitrogen (Stokes and Larsen, 1945). The nature of bacterial protein may be independent of the amino acid composition of the medium (Camien, Salle and Dunn, 1945; Freeland and Gale, 1947), while proteins of organisms grown in the absence of non-essential amino acids can be shown to contain those amino acids. Micro-organisms must therefore be capable of synthesising non-essential amino acids either from other constituents of the nutrient medium or from the intermediates of carbohydrate or fat metabolism. A more detailed study of the growth-promoting effects of various amino acids and their precursors can indicate the pathways for some of these syntheses.

Synthesis of tryptophan

As already mentioned, an exacting strain of *Eberthella typhosa* will not grow on a simple salt glucose medium but grows readily if tryptophan is added to the medium. Tryptophan as a growth stimulant can be replaced by indole (Fildes, 1940*b*). The organism is apparently incapable of synthesising indole, but, if presented with indole, can convert it to tryptophan. Indole was also found to be a growth factor for *Corynebacterium diphtheriæ* in a tryptophan-free medium. Some strains of staphylococcus were found to be capable of the conversion of indole to tryptophan whereas other strains required preformed tryptophan. Such results suggest that indole may be an intermediate in the biological synthesis of tryptophan. A further extension of the metabolic pathway is indicated by the capacity of anthranilic acid to act as a growth factor for some strains of lactic acid bacteria in place of either indole or tryptophan (Snell, 1943). It is therefore probable

that tryptophan is normally synthesised through indole by cyclisation of anthranilic acid.



Anthranilic acid *Indole* *Tryptophan*

This assumption has been strikingly confirmed by several different investigations. That of Fildes (1945) showed that indoleacrylic acid, a growth inhibitor which blocks synthesis of tryptophan from indole, is growth-inhibitory when indole has to be converted to tryptophan but not when tryptophan is already present (see Chapter V for further details). Another type of investigation on tryptophan synthesis has been the study by Tatum and Bonner (1944) of nutritive requirements of X-ray mutants of the mould *Neurospora*.

The normal wild-type strains of *Neurospora* are able to grow on a simple medium containing only inorganic salts, a source of organic carbon, and biotin. After exposure to sublethal doses of X-rays, mutant strains may be isolated which are more exacting in their nutritional requirements than the parent strains. The concept that the specific growth requirements of micro-organisms are a reflection, not of intrinsically different needs, but rather of loss of the capacity to synthesise certain metabolites, has already been mentioned. With the mould *Neurospora* genetic analysis is possible, and the synthesis of essential metabolites has been shown to be gene-controlled, each synthetic deficiency being inherited as if it were associated with the mutation of a single gene (Beadle and Tatum, 1941). Any biosynthesis involves a series of consecutive reactions, each catalysed by a specific enzyme. The production of each enzyme appears to be gene-controlled. Mutation may result in loss of the enzyme essential for any one step in a synthetic series, consequently the number of different mutants affecting a given synthesis is a measure of the minimum number of biochemical steps directly involved

in that synthesis. After exposure to X-rays, strains of *Neurospora* were isolated which could not grow on the original simple medium and required the addition of tryptophan or one of its precursors. One strain could not synthesise anthranilic acid but could, if provided with anthranilic acid, grow and synthesise tryptophan. Another mutant could not convert anthranilic acid to tryptophan but could utilise indole. The strain which could form tryptophan from indole but not from anthranilic acid actually produced anthranilic acid, but the next stage in the synthesis was blocked because of lack of the specific enzyme.

Participation of serine in the synthesis was also demonstrated. Indole was only slowly utilised by a growing culture in the absence of serine, but addition of serine caused an increase in the rate of production of tryptophan which was proportional to the amount of serine added. Other theoretically-possible intermediates were completely inactive. A cell-free enzyme requiring pyridoxal phosphate as coenzyme was prepared from *Neurospora* and found to be capable of synthesising tryptophan from indole and serine (Umbreit, Wood and Gunsalus, 1946). The well-known degradation of tryptophan to indole by *Escherichia coli* (Fildes, 1938) was first assumed to be the reverse of the synthetic process (Tatum and Bonner, 1944). Evidence has now accumulated that argument by analogy was misleading. A cell-free tryptophanase enzyme has been isolated from *E. coli* which catalyses the reaction: tryptophan→indole+pyruvic acid+ammonia. The enzyme can be resolved into an inactive apo-enzyme and its coenzyme, pyridoxal phosphate. Apparently, serine is not an intermediate in this process, since the purified enzyme did not deaminate serine (Wood, Gunsalus and Umbreit, 1947).

Deductions as to fundamental routes for the biosyntheses of amino acids and other essential metabolites made from studies with X-ray mutants of moulds are thus useful pointers to the metabolic routes followed in other micro-organisms, but are not necessarily applicable to all micro-organisms and should be regarded mainly as a guide to more direct investigation. Fungi, like pathogenic bacteria, often show

an inability to synthesise essential metabolites, which may be regarded as a naturally-occurring evolutionary loss correlated with gene mutation (see Chapter VI).

Amino acid antagonisms

Bacillus anthracis will grow very well on a glucose-salt medium containing the following seventeen amino acids: aspartic acid, valine, leucine, alanine, glutamic acid, isoleucine, phenylalanine, lysine, glycine, proline, hydroxyproline, tyrosine, arginine, histidine, cystine, methionine and tryptophan. If, however, valine, leucine or isoleucine is added singly to a mixture of amino acids able to support growth without it, growth is prevented (Gladstone, 1939). The toxic effect of leucine may be counteracted by valine, and *vice versa*; the toxic effect of isoleucine is only overcome if both valine and leucine are added. Other antagonisms were found between threonine and serine and between valine and α-aminobutyric acid. Similar types of antagonistic pairs have been noted with other organisms. Glutamine and aspartic acid are antagonistic for *Lactobacillus casei* (Feeney and Strong, 1942); leucine and methionine are antagonistic for *Proteus morganii* (Porter and Meyers, 1945). Possibly these antagonisms arise because of close relationships between the mechanisms for biological synthesis of the antagonistic pairs, the precursor of one amino acid being able to interfere with formation of another. This view is supported by isolation of a " valine-less " (*i.e.* unable to synthesise valine) X-ray mutant strain of *Neurospora crassa* which was found to require both isoleucine and valine in an optimal ratio of 70-80 per cent. valine to 30-20 per cent. isoleucine (Bonner, Tatum and Beadle, 1943; Bonner, 1946). This apparent double requirement of a single mutant is thought to be due to failure of amination of keto-isoleucine to isoleucine with consequent accumulation of keto-isoleucine. The keto acid may specifically inhibit amination of the closely related keto-valine, so preventing synthesis of valine.

These examples of amino acid antagonisms indicate the complexity of the interpretation of nutritional studies, and suggest the difficulties which have to be met in interpreting

the mode of action of metabolite antagonists. It should be noted that the growth-inhibitory effect of one amino acid is generally dependent upon the failure of a strain to synthesise optimal amounts of another structurally-related amino acid.

Metabolite deficiency and metabolic response

Amino acids are involved in the general protein synthesis of the cell, and so are equally essential for the formation of a vast array of enzymes; consequently, deficiency of any one amino acid is hardly likely to be reflected in a recognisable deficiency of any single enzyme or group of enzymes. A knowledge of the amino acid requirements of micro-organisms only provides information about the nature of the overall metabolism of the cell. Information about the part played by certain enzymes may be obtained by growing micro-organisms in media deficient in a single coenzyme or essential trace metal. The cell may then exhibit a lop-sided metabolism which can provide information, both as to the function of the coenzyme or metal, and also as to the relative importance to the cell of different enzymes with common prosthetic groups or co-enzymes. The nature of the response observed on addition of an essential metabolite to a culture containing suboptimal amounts of that metabolite may also indicate its function in the cell.

Metallic metabolites

In discussion of the individual enzymes of cellular metabolism, attention was drawn to the importance of metals such as iron, zinc, magnesium and manganese as essential constituents of numerous enzyme systems. Relatively little is known about the inorganic salt requirements of bacteria. The earlier literature suggested that no ions other than sodium, potassium, chloride, sulphate and phosphate were necessary for growth, but it is now generally recognised that these conclusions were reached because of the difficulty of freeing a medium of all traces of other ions. Only very rigorous and carefully designed experiments using specially purified materials can give reliable information as to the exact inorganic salt requirements of micro-organisms.

The trace element requirements of only a few micro-organisms have been investigated (Young, Begg and Pentz, 1944). Zinc has been almost completely removed from a medium by extraction with a solution of diphenylthiocarbazone in carbon tetrachloride (Feeney, Lightbody and Garibaldi, 1947). The medium, containing less than 0·004 parts per million of zinc, supported slow growth of *Bacillus subtilis* but the antibiotic, subtilin, normally produced by the strain, was no longer synthesised. As the zinc content of the medium was increased, both growth and antibiotic production increased towards normal. The growth of *Corynebacterium diphtheriæ* can be greatly slowed by reduction of the iron content of the medium. As the iron content of the medium is raised above the minimal requirement, increased growth is accompanied by increased excretion of diphtheria toxin and of porphyrin. Beyond a critical level of iron there is a rapid fall in yield of toxin and porphyrin, but growth rate continues to increase (Pappenheimer and Hendee, 1947). Simultaneously with the fall in porphyrin excretion there appears, in the cell suspension, the characteristic spectrum of cytochrome *b*. Small amounts of cytochrome *a* have also been reported in *C. diphtheriæ*, but no cytochrome *c* (Rawlinson and Hale, 1948). Pappenheimer and Hendee suggest that the cell may be unable, in an iron-deficient medium, to synthesise complete cytochrome *b* but continues to synthesise bits of the molecule which appear in the medium as prophyrin and diphtheria toxin.

Waring and Werkman (1942 *a* and *b*, and 1944) freed media from traces of iron by treating with 8-hydroxyquinoline and extracting the iron-quinoline complex with chloroform. Using these media, they found that *Escherichia coli*, *Aerobacter indologenes* and *Klebsiella pneumoniæ* required 0·02 to 0·03 parts per million of iron for growth. *Pseudomonas pyoyanea* unlike these other three organisms which have incomplete cytochrome systems, normally shows a complete 4-banded cytochrome spectrum and high peroxidase and catalase activities; as might be expected, it required three to four times more iron. When the iron content of the medium was suboptimal, growth of all these organisms was proportional to

the amount of iron present, and the iron content of the cells showed a direct relationship to the concentration of iron in the medium. *Aerobacter indologenes* grown on a medium deficient in iron showed only 5 per cent. of its normal catalase and peroxidase activities. The cytochrome activity, as indicated by cyanide-sensitive aerobic respiration, was normal, but the two weak cytochrome bands visible in normal cells were no longer visible in the iron-deficient organisms. The dehydrogenase activity of the deficient cells was unimpaired except for a loss of formic dehydrogenase and formic hydrogenlyase. It appears that the iron-deficient cells had dispensed with the less essential enzymes dependent on iron, in order to spare the available metal for the more essential cytochrome system. Essentially similar results were obtained with *Aerobacter cærogenes* by Perlman (1945) who utilised cationic exchange resins to free the medium from trace elements.

The nature of glucose metabolism by *Clostridium welchii* depends on the iron content of the medium (Pappenheimer and Shaskan, 1944). As the available iron is reduced, the fermentation changes from a predominantly acetic-butyric acid type, producing large amounts of carbon dixoide and hydrogen, towards a lactic acid type of fermentation which produces little gas. *Cl. welchii* does not possess a cytochrome system but apparently iron plays some essential part in its normal glucose fermentation. Almost complete inhibition of normal glucose fermentation of *Cl. tetani* can be caused by treatment of a washed suspension of normal organisms with $\alpha : \alpha'$-dipyridyl, a reagent which is known to combine with ionic iron but not with the iron of hæmatin enzymes. Azide, which acts as an inhibitor of hæmatin enzymes, has no inhibitory effect on fermentation (Lerner and Pickett, 1945). Earlier observations by Kubowitz (1934) suggest that iron or another heavy metal is an essential part of some enzyme involved in the acetic-butyric acid type of fermentation of *Clostridium butyricum*. High concentrations of carbon monoxide, which are known to inactivate heavy metal catalysts, diverted the fermentation to the production of lactic acid. The commercial conversion of maize mash to acetone and butyl

alcohol by *Cl. acetobutylicum* is also inhibited by carbon monoxide, but in a continuous stream of carbon monoxide a suitable cell suspension can convert glucose to lactic acid (Simon, 1947).

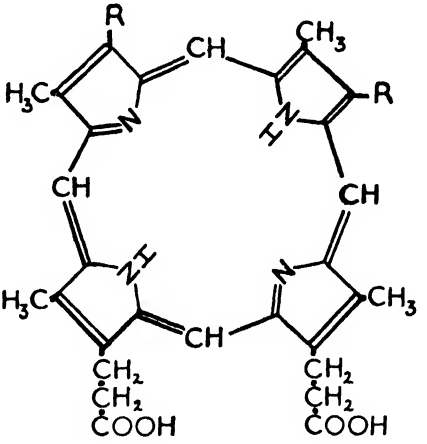
It is apparent from these few results that much useful information on the enzymic function of trace metals is likely to accumulate as methods are developed for the preparation of metal-free media. It hardly needs to be emphasised that any organic compound capable of forming a stable organometallic complex is a potential enzyme inhibitor, and a suitable pattern for chemotherapeutic research.

Organometallic metabolites

A few groups of organisms seem to have lost the capacity to synthesise hæme enzymes from iron. Davis (1917) found that *Hæmophilus influenzæ* (Pfeiffer's bacillus) could not grow in peptone broth unless supplied with two additional growth factors, one of which was thermostable and occurred in blood pigments. Thjötta and Avery (1921) suggested the term " X-factor " for the growth substance contained in blood pigments and the term " V-factor " for the other, thermolabile, growth factor present in tissue extracts. Over a period of years, " X-factor " has been shown to be essential for several other species of the genus *Hæmophilus* and for *Bartonella bacilliformis* and *Pasteurella pestis*, also for some protozoa including several trypanosomes and leishmania (Jiménez, 1940; Rao, 1939; Lwoff, 1938). The X-factor was found by A. and M. Lwoff (1933 *a*, *b*) to be part of the iron-containing pigment of blood; hæmoglobin itself was much less active than the porphyrin pigment hæmatin; other porphyrin pigments such as chlorophyll were inactive, but catalase and plant peroxidase had activity. Lwoff suggests that exacting organisms have lost the capacity to synthesise from simple nutrients the iron-porphyrin enzymes such as cytochrome oxidase, catalase and peroxidase. The iron-porphyrin enzymes are concerned with aerobic metabolism and with the immediate destruction of any hydrogen peroxide formed under aerobic conditions; under anaerobic conditions those enzymes are

likely to be of less importance to the cell. This interpretation was supported by reports that *Hœmophilus influenzæ* did not require X-factor for growth in the absence of oxygen. A careful study by Gilder and Granick (1947) suggests that this may be only partially true since *H. influenzæ* Turner requires a limited supply of hæme even in the complete absence of oxygen.

Lwoff (1933*b*, 1936) found that trypanosomes grown in media containing suboptimal amounts of hæmatin showed a respiratory rate of about one-third normal; the reduced



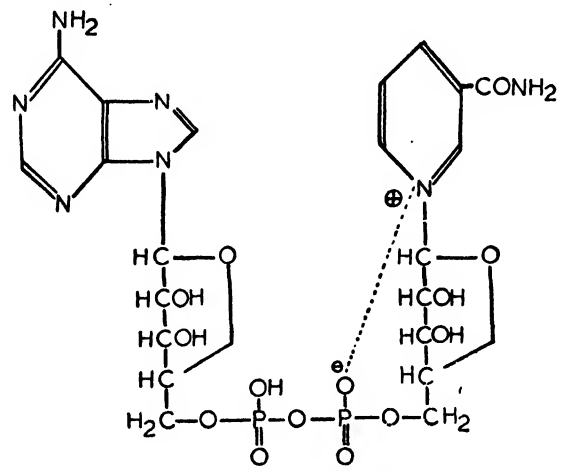
Protoporphyrin	R is $CH{=}CH_2$
Hæmatoporphyrin	R is $CHOH{-}CH_3$
Mesoporphyrin	R is $CH_2{-}CH_3$
Coproporphyrin	R is $CH_2{-}CH_2{-}COOH$

FIG. 14.—Porphyrin skeleton.

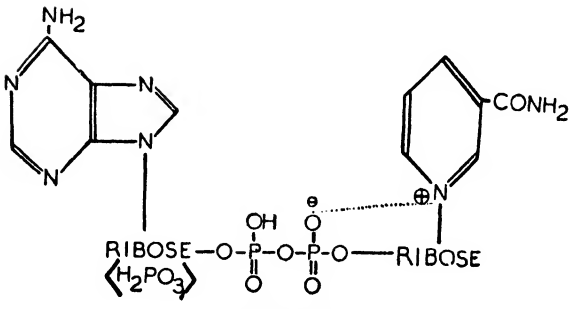
respiration was restored to normal by hæmatin, or by protoporphyrin and iron. Although cytochrome alone could not replace hæmatin, in the presence of suboptimal amounts of blood it caused considerable augmentation of growth of trypanosomes.

The loss of synthetic ability of *H. influenzæ* lies in a failure to synthesise protoporphyrin, since "X-factor" may be replaced by a mixture of iron and protoporphyrin. Various strains differ in their response to other porphyrins. In some strains iron-mesoporphyrin or mesoporphyrin in low concentration supports growth, in others, the vinyl side-chains (see Fig. 14) are essential and compounds such as coproporphyrin,

mesoporphyrin and hæmatoporphyrin fail to support growth or even inhibit growth. Simple esterification of the propionic acid side-chains of hæmatin or protoporphyrin may prevent utilisation (Granick and Gilder, 1946; Gilder and Granick, 1947).



Coenzyme I



Coenzyme II

Nicotinic acid derivatives

Thjötta and Avery (1921) suggested the name " V-factor " for the thermolabile growth factor contained in tissue extracts and required by *Hæmophilus influenzæ*, but were unable to elucidate its nature. It was not until 1936 that the " V-factor " was identified by Lwoff, as di- or possibly triphosphopyridine nucleotide (coenzyme I or II). Coenzyme II is rather less active as a growth factor than coenzyme I and

is possibly used by the organism as a source of coenzyme I, but adenosine, nicotinamide and nicotinic acid are inactive (Lwoff and Lwoff, 1936, 1937*a*; Schlenk and Gingrich, 1942; Gingrich and Schlenk, 1944). All micro-organisms which do not require " V-factor " are believed to synthesise it, either from inorganic salts and a carbon source, or from preformed portions of the molecule such as nicotinic acid and adenine. In other words, coenzymes I and II are essential parts of the enzymic structure of all organisms, but there is considerable variation in the ability of different micro-organisms to carry out the various steps of synthesis. A mixture of nicotinamide, *d*-ribose and adenylic acid cannot replace coenzyme I in promoting the

TABLE 4

Relative growth-promoting effects of Nicotinamide and Nicotinic Acid for different bacteria

Organism	Ratio of activity of amide/acid
C. diphtheriæ	1 : 10
Pr. vulgaris	1 : 1
Staph. aureus	5 : 1
Dysentery bacillus	10 : 1
(certain) Pasteurellæ	∝ (acid ineffective)

Koser, Berkman and Dorfman (1941)

growth of *Hæmophilus influenzæ*, but nicotinamide riboside is able to maintain growth. This organism has apparently lost the capacity to link nicotinamide with ribose, but can perform the other steps in the synthesis of the pyridine nucleotides.

Other organisms which fail to synthesise coenzyme from inorganic salts and carbohydrate usually do so because of their inability to synthesise nicotinic acid or nicotinamide. Nicotinic acid can act as a growth factor for various organisms, but different species differ considerably in their ability to convert nicotinic acid to nicotinamide, and hence in their ability to utilise nicotinic acid in place of nicotinamide. This difference is indicated in Table 4, which shows that *Coryne-*

bacterium diphtheriæ can utilise nicotinic acid ten times better than the amide. A strain of *Leuconostoc mesenteroides* has been found to require nicotinic acid for growth and to be quite unable to utilise nicotinamide (Johnson, 1945). Bearing in mind a possible effect of permeability differences, these results suggest that nicotinic acid itself may play a part in cellular metabolism, and may not simply be a precursor of nicotinamide.

There is also doubt whether the only function of nicotinamide is as a building block for the coenzymes of the pyridine nucleotide dehydrogenases. Symptoms of black-tongue in dogs can be cured by nicotinic acid, but do not respond to intravenous injection of an equivalent quantity of diphosphopyridine nucleotide. Nicotinamide-starved dysentery bacilli responded more favourably to nicotinamide than to an equivalent quantity of disphosphopyridine nucleotide or triphosphopyridine nucleotide (Dorfman, Koser, Horwitt, Berkman and Saunders, 1940; Saunders, Dorfman and Koser, 1941). This difference may, of course, be due to failure of the larger molecules to penetrate the cell wall as readily as nicotinamide.

Knowledge of the importance of pyridine nucleotides as components of the dehydrogenase systems of carbohydrate metabolism, naturally suggests that when organisms are grown on media containing suboptimal amounts of coenzyme, some dehydrogenases would be lacking or deficient. Lwoff and Lwoff (1937*b*) found that during logarithmic growth on a medium containing limited supplies of " V-factor," dehydrogenase activity was normal, but as the growth rate began to decrease (*i.e.* when all available coenzyme had been used) the dehydrogenase activity fell off at the rate of 30 per cent. of total activity per hour. Dehydrogenase activity returned to normal as soon as coenzyme was added. The results are shown in Table 5 together with the change induced by addition of coenzyme to the starved cells. The ability to reduce methylene blue in presence of any one substrate was taken to indicate dehydrogenase activity. Similar results have been obtained for *Proteus vulgaris* by Morel (1941).

When an organism is presented with a source of nicotinamide or nicotinamide riboside it may build either coenzyme I or coenzyme II ; the nature of the coenzyme formed probably depends on the type of metabolism which the organism finds itself obliged to adopt, in other words it depends on the other nutrients present in the medium. Aerobic oxidation of glucose seems to use up coenzyme II, aerobic oxidation of pyruvate uses coenzyme I (Lwoff and Lwoff, 1937*b* ; Morel, 1941). *Salmonella paratyphi A* cannot utilise carbohydrate without

TABLE 5

Dehydrogenase activity of "coenzyme-starved" H. influenzæ

Substrate	Dehydrogenase Activity in Peptone + Glucose or Hexose monophosphate	
	Without added coenzyme	With added coenzyme
Glucose	0	+
Hexose monophosphate	0	+
Pyruvate	0	+
Fumarate	0	+
Malate	0	+
Alcohol	0	+
Asparagine	0	+
Valine	0	+
Succinate	+	+
Lactate	+	+

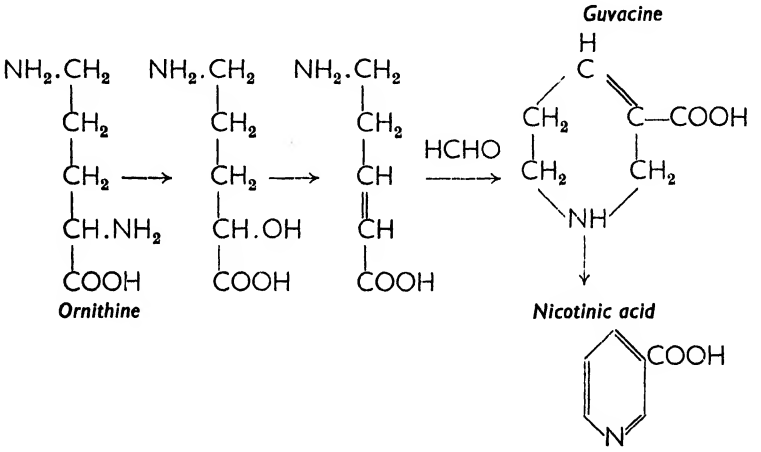
Adapted from Lwoff and Lwoff (1937*b*)

added nicotinic acid. The organism can grow in an amino acid-peptone medium without any supply of nicotinic acid, but growth is completely inhibited if a fermentable carbohydrate is added to the medium, unless nicotinic acid is added at the same time (Kligler and Grossowicz, 1941). This dependence of growth factor requirements on the nature of the medium has been frequently observed and will arise again when we come to analyse growth inhibition by sulphonamides and other bacteriostatic drugs.

As with other growth factors, nutritional requirements are not absolute and strains which are initially exacting towards

nicotinamide may be trained to dispense with it (Koser and Wright, 1943). The trained organisms continue to synthesise " V-factor " and must therefore have developed the capacity to synthesise nicotinamide.

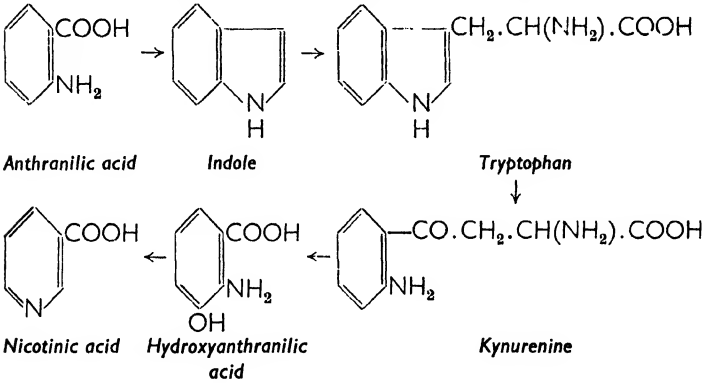
Little is known of the route by which nicotinic acid is synthesised by those organisms which do not require an external source. Ornithine may be deaminated to ω-amino-propylideneacetic acid and cyclised by reaction with formaldehyde to give guvacine as indicated in the following scheme :—



Guvacine or hexahydronicotinic acid have been found to replace nicotinamide in the nutrition of *Staph. aureus* and *Proteus vulgaris* (Euler, Högberg, Karrer, Salomon and Ruckstühl, 1944).

An alternative route for nicotinic acid synthesis has been suggested by a study of " nicotinic acid-less " mutants of *Neurospora*. Genetic analysis of the induced mutants suggested that nicotinic acid synthesis might be blocked at any one of three separate loci. Possible precursors of nicotinic acid, such as pyridine, β-picoline, γ-picoline, piperidine, piperidine-3-carboxylic acid (hexahydronicotinic acid), trigonelline, ornithine, proline, α-amino-*n*-valeric acid and α-amino-*n*-caproic acid were all unable to replace nicotinic acid for growth of the mutant strains and were, therefore, not likely intermediates on the biosynthetic pathway blocked

by mutation. Two of the mutant strains were, however, able to convert tryptophan or kynurenine to nicotinic acid. Two other genetically-distinct "nicotinic acid-less" strains were unable to use tryptophan or kynurenine but, when supplied with minimal quantities of nicotinic acid, accumulated hydroxyanthranilic acid in the medium (Bonner, 1948; Mitchell and Nyc, 1948). These results suggest that a biosynthetic route (formulæ below) for nicotinic acid synthesis may extend from anthranilic acid, through indole and tryptophan (p. 107) to kynurenine, with some still unrecognised further stages through hydroxyanthranilic acid.



Structural specificity of substituted pyridines

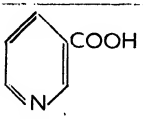
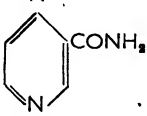
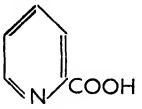
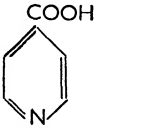
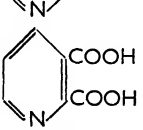
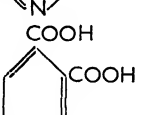
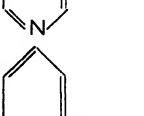
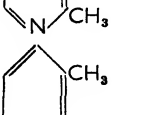
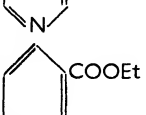
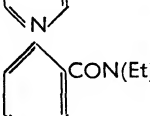
As already indicated, growth requirements are in no way absolute and depend upon the environment in which a test is made, so that it is impossible to classify pyridine derivatives as active, partially active and completely inactive as growth factors. It is possible, however, to take a broader view and to consider pyridine derivatives as potential precursors of essential coenzymes which may or may not, according to the metabolic abilities of a micro-organism, be converted to a form suitable for incorporation into essential coenzymes. An examination of the data collected by various workers on the growth-promoting activity of pyridine derivatives, leads to the general conclusion that unless the pyridine ring is substituted in the 3 position, as in nicotinic acid, it cannot be utilised. The two

isomeric acids, picolinic and *iso*nicotinic, fail to promote growth (Table 6). Introduction of a second substituent into the ring of nicotinic acid usually produces an inactive compound, but some micro-organisms can use pyridine-2 : 3-dicarboxylic acid, presumably because they are capable of converting the dicarboxylic acid to nicotinic acid. This observation raises an interesting question which cannot be answered until methods are worked out for the complete synthesis of coenzymes I and II. It is not clear whether the inactivity of some compounds is due to the inability of micro-organisms to build them up into a coenzyme-like structure, or is due to a functional failure of a coenzyme built on a slightly different pattern from the normal structure. Where inactivity is confined to a few species of micro-organisms and the same compound can be utilised by other species, the failure is presumably in the initial synthesis of coenzyme. Where all species fail to grow when a nicotinic acid analogue is substituted for nicotinic acid, the failure may be functional; in other words, an enzymically inactive analogue of coenzyme I or II may be synthesised but not utilised. Some of the results of testing pyridine derivatives as growth factors are summarised in Table 6.

Purines and pyrimidines

The ability to synthesise the adenylic acid portion of phosphopyridine nucleotides seems to be less frequently lost in parasitic organisms than the ability to synthesise the nicotinic acid part of the molecule. This difference may be related to the very wide biological importance of adenine and closely related purines. Adenine is not only essential for synthesis of "V-factors," but is also a component of many other enzyme systems, for example the flavin adenine nucleotides; and in the form of adenosine di- or triphosphate, it plays a fundamental role in energy transfer. In addition, adenine and the related purine guanine form an important part of bacterial nucleoprotein (Stahl, Pennel and Huddleston, 1939). It is not surprising therefore that a parasitic organism hesitates for strategic reasons before lowering its tariff barriers

Pyridine derivatives as growth factors

Substance		Organism			
Name	Formula	*Staph. aureus*	*Shigella dysenteriæ*	*Proteus vulgaris*	*Lacto-bacillus arabinosus*
Nicotinic acid		+	+	+	+
Nicotinamide		+	+	+	+
Picolinic acid		−	−	−	−
Isonicotinic acid		−	−	±	...
Quinolinic acid		−	+	±	...
Cinchomeronic acid		...	−	...	...
α-Picoline		−	...	...	...
β-Picoline		−	−	+	...
Ethyl nicotinate		+	+	+	−
Coramine		−	+	+	...

Adapted from Knight (1945).

and allowing foreign-manufactured adenine to displace the home-made product, even though the imported article may be economically desirable.

A strain of *Strep. hæmolyticus* has been found to require both nicotinic acid and adenylic acid or other purine (*i.e.* both portions of coenzymes I and II) at low carbon dioxide pressures (Pappenheimer and Hottle, 1940). Other strains of this organism could not utilise adenine, but grew on xanthine, guanine, hypoxanthine, guanosine or adenosine (Wilson, 1945). Various lactic acid bacteria may also fail to grow under certain conditions in the absence of adenine or guanine (Snell and Mitchell, 1941), but more usually these purines have been found to have growth-stimulating rather than growth-initiating effects (Feeney and Strong, 1942; Snell and Mitchell, 1942). The distinction is perhaps somewhat academic, since a substance which promotes growth under one set of conditions may initiate growth under another. In either case, the nutrient is playing an essential part in cell metabolism.

Purines have been found to be essential metabolites for protozoa and for fungi as well as for bacteria, another indication of the fundamental unity of intermediate metabolic pathways in different types of living cells (Kidder and Dewey, 1945; Robbins and Kavanagh, 1942).

Up to the present, studies on purine-starved organisms have not implicated any particular enzymic systems, but the growth response of deficient "adenine-less" *Neurospora* was greater with coenzyme I than with adenine or adenosine (Pierce and Loring, 1945). Purine deficiency has, however, received relatively little attention as yet, and it is possible that further study will indicate specific enzymic deficiencies in purine-starved cells. Suggestions have been repeatedly made during recent years that purines are concerned with *p*-aminobenzoic acid metabolism and therefore with the mode of action of sulphonamides. This aspect of purine function will be more fully discussed in Chapter V.

So far as we know from the scanty data available, bacterial nucleic acid, like yeast and animal nucleic acid, is built from pyrimidines as well as purines. Levene (1904) identified

thymine and uracil as components of nucleic acid from *Mycobacterium tuberculosis*. Later Johnson and Brown (1922) reinvestigated the problem and identified thymine and cytosine but could not identify uracil. The nucleic acid from the closely related organism *Mycobacterium phlei* contained, in addition to adenine and guanine, the pyrimidines cytosine and uracil but no thymine (Coghill, 1931). The nucleic acid from *Corynebacterium diphtheriæ* contained all three pyrimidines (Coghill and Barnés, 1932). Recent work by Sevag and his colleagues has indicated that the nucleic acid of *Strep. hæmolyticus* is a mixture of the desoxyribose and ribose types (Sevag, Smolens and Lackman, 1940 ; Sevag and Smolens, 1941).

Uracil has been found essential for anaerobic culture of *Staph. aureus* but is probably synthesised by the organism under aerobic conditions (Richardson, 1936). Various exacting strains of lactobacilli respond to thymine and cytosine as well as to uracil (Snell and Mitchell, 1941).

The assumption that micro-organisms requiring pyrimidines build these bases up partly to the nucleic acid form is supported by the nature of the growth response of " pyrimidine-less " X-ray mutants of *Neurospora*. The pyrimidine nucleosides uridine and cytidine and the nucleotides uridylic acid and cytidylic acid were from ten to sixty times as active as the two most active pyrimidines uracil and orotic acid (Loring and Pierce, 1944). Stokes (1944) suggested that folic acid may participate in the synthesis of pyrimidines, since high concentrations of thymine or its nucleoside can replace folic acid in the nutrition of *Streptococcus fæcalis*. Other pyrimidines or pyrimidine analogues containing the 5-methyl group can replace thymine, a sulphur analogue of thymine (5-methyl-2-oxy-4-thiopyrimidine) being as active as thymine itself (see Table 7) (Hitchings, Falco and Sherwood, 1945). Purines have been implicated as products of the metabolic system of the cell which requires *p*-aminobenzoic acid, and *p*-aminobenzoic acid is a component of folic acid ; in other words, failure to utilise *p*-aminobenzoic acid may result in failure of the whole nucleic acid synthesising system (see Chapter V for further discussion).

As more complete information is collected on the metabolism of pyrimidine-starved organisms and on the metabolic effects induced by added pyrimidines, functions additional to those of nucleoprotein synthesis may be allocated to these bases.

TABLE 7

Response of L. casei *to various pyrimidines*

Substance	Concentration, mg./10 ml.	Effect of Pyrimidine in Various Media. Change of Acid Titre per cent.		
		Unsupplemented	With thymine, 1 μg./ml.	With folic acid, ·0014 μg./ml.
Thymine	0·01	+500	0	+20
5-Methyl cytosine	0·1	+200	...	...
5-Methyl isocytosine	0·2	+300	0	+10
5-Methyl-2, 4-diamino-pyrimidine	1·0	+300	−20	+20
5 - Methyl - 2 - thio - 4 - oxy-pyrimidine	0·05	−30	+10	+10
5 - Methyl - 2, 4 - dithio-pyrimidine	0·25	+20	−25	0
5 - Methyl - 2 - oxy - 4 - thio-pyrimidine	0·004	+300	+15	0
5 - Methyl - 2, 4-dioxy - 6 - imino-pyrimidine	1·0	−10	0	0
5, 6-Dimethyl-2, 4-dioxy-pyrimidine	1·0	0	0	0
2, 5-Dimethyl-4-oxy-pyrimidine	1·0	+30	+10	+10
5-Hydroxy uracil	1·0	−50	−70	−75
5-Amino uracil	1·0	−50	−25	−55
5-Carbamido uracil	1·0	−50	−55	0
5-Chloro uracil	1·0	+45	−93	+20
5-Bromo uracil	0·2	+20	−40	+15
5-Iodo uracil	1·0	0	−40	−20
5-Nitro uracil	0·2	0	−14	−65

From Hitchings, Falco and Sherwood (1945).

The special case of the enzymic function of the " pyrimidine half " of thiamin (vitamin B_1) is treated separately.

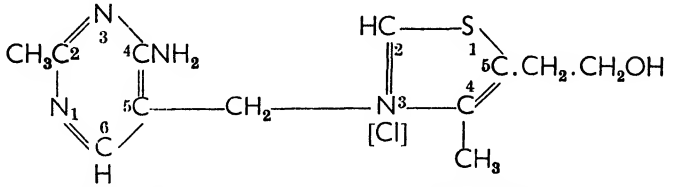
Thiamin (vitamin B_1 or aneurin)

Thiamin is an essential metabolite for bacteria, fungi, protozoa, plants and animals, and has been identified as a

component of a number of enzyme systems, particularly those concerned with pyruvate metabolism (p. 73).

From a study of the change in metabolism following addition of thiamin to thiamin-starved *Staph. aureus*, Hills (1938) concluded that anaerobic dismutation of pyruvate to lactate, acetate and carbon dixoide depended on an enzyme system involving thiamin. Later Smyth (1940) extended these results by showing that oxaloacetate could to some extent replace thiamin, suggesting that one of the functions of thiamin may be to catalyse the formation of oxaloacetate from pyruvate and carbon dioxide. When thiamin was added to thiamin-deficient *Propionibacterium pentosaceum* there was a considerable lag period (120 mins.) in the commencement of anaerobic production of carbon dioxide from pyruvate. Addition of diphosphothiamin (co-carboxylase) under similar conditions cut the lag period to thirty minutes. Presumably the thiamin had to be converted to diphosphothiamin before becoming functional in anaerobic pyruvate metabolism (Silverman and Werkman, 1939*b*). An accumulation of pyruvic acid in aerobic glucose utilisation of thiamin-deficient organisms has been noted; the addition of thiamin to the cells stimulated aerobic but not anaerobic glucose utilisation (Kligler, Grossowicz and Bergner, 1943).

For convenience of discussion the thiamin molecule is regarded as made up of two "halves." The pyrimidine half and the thiazole half, numbered as indicated below.



Pyrimidine Half *Thiazole Half*

Micro-organisms can be divided into four groups according to their ability to synthesise thiamin :—

(1) Those which synthesise thiamin from simpler constituents of the medium.

(2) Those which can synthesise thiamin if supplied with one " half " of the molecule.

(3) Those which can synthesise thiamin only if supplied with both " halves " of the molecule.

(4) Those which cannot synthesise thiamin even if supplied with both " halves."

These four groups represent successive stages in loss of synthetic ability following upon the adoption of a parasitic existence. It must be remembered that this type of classification is a convenience rather than an actuality, and loss of synthetic ability is a finely-graded process capable of alteration in an altered environment. Certain gonococci may represent a fifth group with even less synthetic ability, since they are unable to utilise thiamin itself but require thiamin pyrophosphate (co-carboxylase) preformed in the medium. Thiamin in this case even inhibits growth by competition with co-carboxylase (Lankford and Skaggs, 1946). Although many strains of micro-organisms have lost the ability to synthesise thiamin, none, so far as is known, has at the same time lost the need for thiamin as a component of its metabolic system.

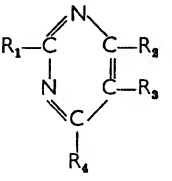
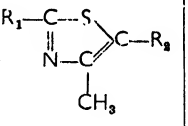
For growth of *Staph. aureus*, a mixture of the pyrimidine and thiazole " halves " can replace the intact molecule, and the organism must be presumed to possess an enzyme capable of uniting the two parts. Striking confirmation of the necessity of the complete molecule rather than the constituent parts for metabolic function is provided by a " thiamin-less " mutant of *Neurospora* which has been found to be able to synthesise both " halves " but cannot dispense with thiamin because it is unable to join the two together (Tatum and Beadle, 1945; Tatum and Bell, 1946). *Staph. aureus* is capable of utilising some pyrimidines and thiazoles closely related to the natural " halves " of thiamin as is indicated in Table 8. The response of certain protozoa to various pyrimidines and thiazoles is summarised by Lwoff (1947).

The question naturally arises, does the micro-organism convert all the active analogues of the natural components to a common coenzyme, or can coenzymes of slightly different

structures be synthesised and utilised? Insufficient information is available to give a definite answer; in this, as in other similar cases, a study of the specificity requirements for coenzyme action in isolated enzyme systems would be instructive. There are indications that in pea roots, at least,

TABLE 8

Effect of structure on availability of pyrimidines and of thiazoles as growth factors for Staph. aureus

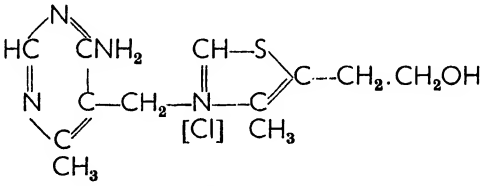
Pyrimidines					Thiazoles		
General Structure 					General Structure 		
R_1	R_2	R_3	R_4	Response	R_1	R_2	Response
CH_3	NH_2	$CH_2.NH_2$	H	++++	H	$CH_2.CH_2.OH$	++++
CH_3	NH_2	$CH_2.OH$	H	++++	H	$CH_2.CH_2OAc$	+++
CH_3	NH_2	$CH_2.NH.CSH$	H	++	H	$CH_2.CHOH.CH_3$	++
CH_3	OMe	$CH_2.NH_2$	H	−	H	$CH_2.CH_2.CH_2OH$	++
CH_3	NH_2	$CH_2.CONH_2$	H	−	H	$-CH=CH_2$	+
CH_3	OH	$CH_2.NH_2$	H	−	H	$CHOH.CH_3$	−
CH_3	OH	$CH_2.OH$	H	−	CH_3	$CH_2.CH_2OH$	−
CH_3	NH_2	CH_3	H	−	OH	$CH_2.CH_2OAc$	−
CH_3	OH	NH_2	CH_3	−	H	$CH_2.CH_3$	−
CH_3	OH	H	NH_2	−	H	CH_3	−
OH	NH_2	H	H	−	H	H	−

Some plants and micro-organisms are less exacting and can use some of the compounds not available to *S. aureus* (Knight, 1945). Pyrimidines were tested in presence of excess of the "thiazole half" and *vice versa*. (Data from Knight and McIlwain, 1938).

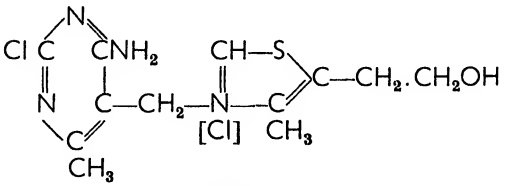
thiazole analogues may be functionally active, although thiamin itself is not synthesised; in other words, a functionally-active analogue of thiamin may be synthesised (Bonner and Buchman, 1938).

Table 8 giving the relation of structure to growth response does not indicate fully the extent of the response. In the

presence of the pyrimidine half, the two thiazoles 4-methyl-5-γ-hydroxypropylthiazole and 4-methyl-5-β-hydroxypropylthiazole are very much less active than the " natural " thiazole 4-methyl-5-β-hydroxyethylthiazole. Two thiamin analogues isothiamin and chlorothiamin (structures below) possessed about $\frac{1}{10,000}$, of the activity of thiamin for *Staph. aureus* (Knight and McIlwain, 1938). These results may be regarded as due to differences in the availability of the compounds for the synthesis of coenzymes or to the relative inactivity of the coenzymes which could be synthesised. The process may be carried one stage further; an analogue can be synthesised which is not simply inactive but instead is actively growth inhibitory, either because it prevents synthesis of the natural coenzymes, or because it is inadvertently utilised by the organism to synthesise a coenzyme analogue which is an enzyme inhibitor. These points will be considered more fully (p. 219) in discussion of competitive inhibition as an explanation of the bacteriostatic action of the thiamin analogue pyrithiamin (Woolley and White, 1943*b*).



ISO-thiamin



CHLORO-thiamin

Riboflavin

Riboflavin is a component of a number of enzyme systems, particularly those concerned with cellular oxidation (Chapter II). As might be expected, it has been found to be an essential

metabolite for micro-organisms as well as for more highly organised life. Where intracellular synthesis is inadequate or where synthetic capacity has been lost following upon a period of parasitic life, riboflavin acts as an essential growth factor or growth stimulant until capacity to synthesise the vitamin is recovered. Loss of synthetic capacity has been most frequently reported among hæmolytic streptococci and lactic acid bacteria (Woolley and Hutchings, 1940; Schuman and Farrell, 1941; Snell and Strong, 1939 *a* and *b*). Actual synthesis of riboflavin has been found in various fungi, moulds and bacteria, including *Mycobacterium tuberculosis*, *Staph. aureus*, *Corynebacterium diphtheriæ* and *Clostridium butyricum* (Warburg and Christian, 1933; Boissevain, Drea and Schultz, 1938; Evans, Handley and Happold, 1939; Peterson and Peterson, 1945; see also Knight, 1945). The extent of riboflavin synthesis is much influenced by the composition of the medium (Mayer and Rodbart, 1946).

Although the enzymic function of riboflavin is fully recognised, there is little evidence available as yet of impairment of any one specific metabolic capacity in intact riboflavin-starved micro-organisms. This may be related to the tendency shown by the majority of micro-organisms to synthesise riboflavin when transferred to a riboflavin-free medium. Adler and Euler (1934) found that riboflavin stimulated the oxygen uptake of riboflavin-deficient lactic acid bacteria and that the additional oxygen uptake was not inhibited by cyanide, showing that it did not go through the cytochrome system. When *Clostridium acetobutylicum* was grown on an iron-deficient medium, riboflavin synthesis increased, suggesting that riboflavin was taking the place of an enzyme system dependent on iron (Tanner, Vojnovich and van Lanen, 1945; Hickey, 1945).

Nutritional requirements for riboflavin are dependent on the nature of the medium; thus, propionic acid bacteria require riboflavin if grown on a medium with ammonium salts as source of nitrogen, but if grown on an amino acid medium no additional growth factor is required (Wood, Andersen and Werkman, 1938). Some strains of lactic acid bacteria when

grown on an amino acid glucose medium require thiamin *or* riboflavin but not both (Wood, Geiger and Werkman, 1940).

Various structural analogues of riboflavin have been tested for their ability to replace the vitamin in bacterial or animal nutrition. Some, as indicated in Table 9, can replace the natural product to a limited extent, others have

TABLE 9

Activity of riboflavin analogues as growth promoters and as coenzymes in isolated systems

Structural Type	Growth Response		Coenzyme Function in Cell-free Enzyme Preparation
	Lactobacillus casei	Rat	
6 : 7-dimethyl-9 (1′*d*-ribityl)	++++	++++	++++
7-methyl-9 (1′*d*-ribityl)	++	++++	++++
6-methyl-9 (1′*d*-ribityl)	+	++++	+++
6-ethyl-7-methyl-9 (1′*d*-ribityl)	+++	++++	−
6 : 7-dimethyl-9 (1′*d*-arabityl)	± *	+	−
6 : 7-dimethyl-9 (1′*l*-arabityl)	± *	+	++
6-ethyl-7-methyl-9 (1′*l*-arabityl)	± *	+	...
6 : 7-dimethyl-9 (1′-sorbityl)	−	−	...
5 : 6-benzo-9 (1′*d*-ribityl)	± *	−	...
5 : 6-dimethyl-9 (1′*d*-ribityl)	± *	Inhibitor	...
6 : 7-dichloro-9 (1′*d*-ribityl)	Inhibitor	...	...
6 : 7-dimethyl-9-galacto	...	Inhibitor	...

* Positive response in presence of sub-optimal amounts of riboflavin.

Data from :—Kuhn and Rudy (1936 *a* and *b*); Kuhn, Vetter and Rzeppa (1937); Kuhn, Weygand and Möller (1943); Snell and Strong (1939*b*); Möller (1940); Sarett (1946); Emerson, Wurtz and Johnson (1945); Emerson and Tishler (1944).

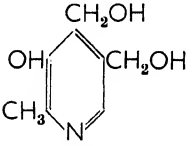
no activity, and others are actively growth-inhibitory. Activity can be related to some extent to coenzyme function, since Kuhn has tested a number of structural analogues on an isolated enzyme system (Kuhn and Rudy, 1936*b*; *cf.* Snell and Strong, 1939*b*; Möller, 1940).

If the sugar residue of riboflavin is removed altogether and replaced by a methyl group as in 6 : 7 : 9-trimethylisoalloxazine, most of the growth-promoting activity disappears and at

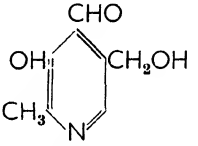
higher concentrations the compound acts as a growth inhibitor for *Lactobacillicus casei*. The significance of the data given above can be more fully appreciated and their chemotherapeutic implications better elaborated, after a discussion of competitive inhibition in enzyme systems.

Pyridoxin, pyridoxal, pyridoxamine

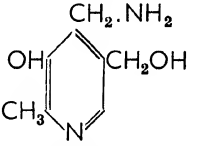
Pyridoxin (vitamin B_6) was first recognised as an essential metabolite in animal nutrition, and later found to be essential for various strains of bacteria, moulds, fungi and plants. Snell (1942) found that some bacteria responded less readily to pyridoxin than to a compound formed by autoclaving pyridoxin with cystine and glycine. Later, after the synthesis of pyridoxin and various related compounds, he found that for some strains of *Strep. lactis* the derivatives pyridoxal and pyridoxamine were 5000 to 8000 times as effective as pyridoxin



Pyridoxin



Pyridoxal



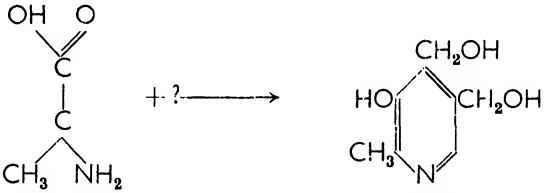
Pyridoxamine

itself (Snell, 1944*b*). All three compounds exist in various types of cells (Snell, 1945*a*) and can probably be interconverted by most organisms. Why pyridoxal and pyridoxamine should be so much more active than pyridoxin for *Strep. lactis* has not been fully explained. With *Lactobacillus casei*, pyridoxal is about 1500 times as active as pyridoxin, and pyridoxamine only three to ten times as active. This difference, which can be used to distinguish between the three compounds (Snell and Rannefeld, 1945), may be due to differences in enzymic requirements of different cells, or it may be a permeability effect. Some micro-organisms, for example certain strains of lactic acid bacteria, grow well without added pyridoxin and have been shown to synthesise more than enough for their own requirements; others, such as *Leuconostoc mesenteroides* and *Staph albus*, can grow slowly in absence of added pyridoxin but do not produce sufficient to allow maximal growth; for

these organisms pyridoxin acts as a growth stimulant (Bohonos, Hutchings and Peterson, 1942; Vilter and Spies, 1940).

As already indicated (Chapter II) pyridoxal has been found to be an essential component of enzymes concerned with amino acid decarboxylation and with transamination, and therefore with amino acid synthesis. These conclusions are amply confirmed by study of the metabolism of pyridoxin- or pyridoxal-starved cells and by the metabolic response induced by addition of these compounds.

Strep. fæcalis will grow in the absence of pyridoxin if sufficient alanine is present in the medium (Snell and Guirard, 1943). The *l*-isomer of alanine (that occurring in protein) was only about one-sixth as active as the *d*-isomer which may function as a precursor of pyridoxin as shown in the accompanying scheme (Snell, 1945*b*; Shive and Shive, 1946).

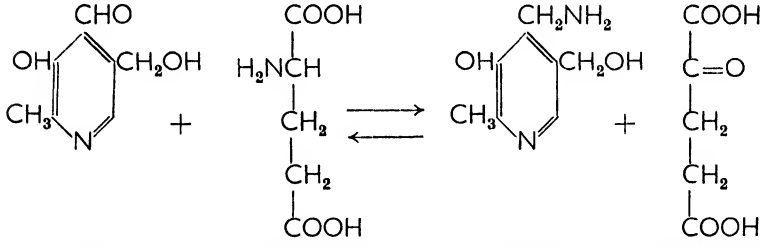


d-alanine *pyridoxin*

Cells grown in the presence of excess alanine and sub-optimal amounts of pyridoxin provided useful material for the investigation of pyridoxin function and behaved as pyridoxin-starved cells. Their rate of glycolysis was normal and was not altered by addition of pyridoxin, but the rate of decarboxylation of tyrosine was very much below normal and was restored to normal by pyridoxal and, to a lesser extent, by pyridoxin. With *Strep. lactis R.*, for which pyridoxin had low growth-promoting activity, addition of pyridoxin to starved cells had little effect on tyrosine decarboxylase activity, while pyridoxal was highly active. In cultures which had been dried, pyridoxal was no longer effective, but full restoration of decarboxylase activity followed addition of phosphorylated pyridoxal or pyridoxal plus adenosine triphosphate (Gunsalus and Bellamy, 1944 *a* and *b*; Gunsalus, Bellamy and Umbreit, 1944; Bellamy and Gunsalus, 1945).

Convincing evidence has now been provided showing that pyridoxal phosphate is an essential coenzyme for four bacterial decarboxylases which act on tyrosine, lysine, arginine and ornithine (Gale and Epps, 1944; Baddiley and Gale, 1945; Gale, 1946; Umbreit, Bellamy and Gunsalus, 1945; Karrer and Viscontini, 1947 *a* and *b*; Gunsalus and Umbreit, 1947). The conditions necessary for production of the protein portion (apo-enzyme) of tyrosine decarboxylase have been investigated using pyridoxin-deficient media containing alanine (Bellamy and Gunsalus, 1945). The concentration in the medium of nicotinic acid, alanine, folic acid and purines, but not of thiamin, influenced the amount of apo-enzyme formed by the growing cells.

The decarboxylases are by no means universal cellular enzymes, and are probably much less important from the point of view of fundamental intermediary metabolism than the transaminases. The suggestion that pyridoxal is involved in transamination came from the observation of Snell (1945*c*), that on heating together glutamic acid and pyridoxal the following reaction took place. The same type of reaction



occurred with other amino acids and pyridoxal or with keto acids and pyridoxamine. Cell-free bacterial and animal extracts were found to catalyse transamination between certain amino and keto acids. The part played by pyridoxal in this reaction was confirmed by an examination of the effect of adding pyridoxal or pyridoxamine to pyridoxal-starved cultures of *Strep. fæcalis*. The starved cells, grown in the presence of excess alanine, were almost devoid of transaminase activity, but addition of pyridoxal resulted in a rapid development of

a glutamate-aspartate transaminase system. The nature of the system was fully confirmed by demonstration of the conversion of added pyridoxamine into pyridoxal phosphate and by the activation of a cell-free enzyme preparation by either pyridoxamine phosphate or pyridoxal phosphate (Lichstein, Gunsalus and Umbreit, 1945; Umbreit, O'Kane and Gunsalus, 1946; Schlenk and Fischer, 1947).

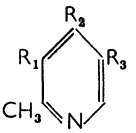
The relative importance of the transaminase and decarboxylase functions of pyridoxal is indicated by the observation that cells growing at almost their normal rate in a suboptimal concentration of pyridoxin showed greatly reduced decarboxylase activity, but little or no reduction in transaminase activity. Transamination was not reduced until the pyridoxin content of the medium was considerably lower, when growth itself was also decreased (Lichstein, Gunsalus and Umbreit, 1945; Cohen and Lichstein, 1945). This provides an excellent example of the way in which a less essential enzyme (decarboxylase) can be dispensed with so that metabolites may be spared for their more essential functions.

There exists a close connection between pyridoxin requirements, carbon dioxide tension and amino acid requirements, which indicates that pyridoxin is involved in the synthesis of a number of amino acids through carbon dioxide fixation. At atmospheric carbon dioxide tension, *Strep. hæmolyticus* will not grow in absence of pyridoxin, but at higher carbon dioxide tensions pyridoxin is unnecessary (Pappenheimer and Hottle, 1940). In the presence of excess carbon dioxide, pyridoxin can replace phenylalanine, tyrosine or arginine in the nutrition of *Lactobacillus arabinosus*, but at atmospheric carbon dioxide tension the omission of any one of these amino acids results in decreased growth which is not restored by additional pyridoxin (Lyman, Moseley, Wood, Butler and Hale, 1947). A similar effect has been found with aspartic acid in *Strep. fæcalis*. Neither of these organisms possessed decarboxylases for the amino acids in question, so that growth must have been related to some reaction other than to decarboxylation. *L. arabinosus*, *L. casei*, and *L. delbruckii* all showed less exacting nutritional

requirements with respect to amino acids when pyridoxamine replaced pyridoxin in the medium (Stokes and Gunness, 1945). In *Neurospora*, pyridoxal phosphate acts as coenzyme in the synthesis of tryptophan from indole and serine; the enzyme catalysing the reaction has been prepared in a cell-free state (Umbreit, Wood and Gunsalus, 1946). In *Escherichia coli* the analogous reaction, transformation of tryptophan to indole plus pyruvic acid and ammonia, is catalysed by another

TABLE 10

Relation of structure of pyridoxin analogues to growth-promoting effect for Lactobacillus arabinosus

Structural Type 			Relative Activities.
R_1	R_2	R_3	
OH	CH_2OH	CH_2OH	1·0 (pyridoxin)
OH	CH_2OAc	CH_2OAc	0·8 to 1·0
OH	CH_2Br	CH_2Br	0·6 to 0·8
OH	CH_2OEt	CH_2OH	0·3
OH	CH_2OMe	CH_2OH	0·3 to 0·4
OH	CH_3	CH_2OH	0·03
OH	CH_3	CH_3	0
OAc	CH_2OAc	CH_2OAc	0
NH_2	CH_2OH	CH_2NH_2	0
OH	CH_2OH	COOH	0

Data from Bohonos, Hutchings and Peterson (1942).

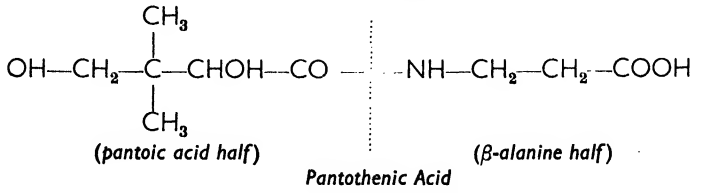
enzyme which also utilises pyridoxal phosphate as coenzyme (Wood, Gunsalus and Umbreit, 1947; see also p. 109). Pyridoxin also appears to be associated with synthesis or utilisation of thiamin (Stokes, Foster and Woodward, 1943).

Structural analogues of pyridoxin show varying degrees of activity as indicated in Table 10.

Pantothenic Acid

Pantothenic acid was first recognised as a growth factor for yeast and animals, and was identified, after isolation

from liver, as $\alpha : \gamma$-dihydroxy-$\beta : \beta$-dimethylbutyryl-β-alanine (Williams, 1943). A few micro-organisms, for example *Lacto-*



Pantothenic Acid

bacillus casei and certain exacting strains of *Corynebacterium diphtheriæ*, require the intact molecule and are unable to unite the two " halves " of the molecule if these are added to the medium (Evans, Handley and Happold, 1939; Evans, Happold and Handley, 1939; Happold, 1940). Other less exacting organisms, such as *Strep. hæmolyticus*, can synthesise pantothenic acid if supplied with the component halves. Some strains of streptococci and *Clostridium septicum* have still greater synthetic powers and can synthesise the β-alanine half if supplied with the pantoic acid half (Woolley, 1939; Ryan, Ballentine, Stolovy, Corson and Schneider, 1945). Non-exacting strains of *Corynebacterium diphtheriæ* may fail to synthesise β-alanine, but if supplied with this component can grow without pantothenic acid (Mueller and Cohen, 1937).

These results are closely reminiscent of the varied capacities existing among different micro-organisms for the synthesis of the two halves of the thiamin molecule. As with other essential metabolites, variation of structure may produce either a compound with lower growth-promoting activity, or one which is completely inactive, or even a compound which is growth-inhibitory (Snell, 1946; see also Chapter V).

Experiments on the effect of addition of pantothenic acid to " starved " organisms suggested that it might be concerned with pyruvate metabolism (Dorfman, Berkman and Koser, 1942; Hills, 1943). With deficient organisms using pyruvate as substrate, both oxygen uptake and anaerobic utilisation of pyruvate were greatly stimulated by added pantothenate; with glucose these effects were not apparent. Fermentation of pantothenate-deficient yeast cells was stimulated by added pantothenic acid and was accompanied by " binding " of the

free acid by the cells; but addition of pantothenate to a cell-free yeast juice had no effect on the rate of glucose phosphorylation or fermentation (Teague and Williams, 1942).

Pantothenic acid is widely distributed and frequently occurs in cells in a " bound " or insoluble form. *Coenzyme A*, a coenzyme of general occurrence has been found to contain pantothenic acid, and probably accounts for a large part of the " bound " pantothenic acid of cells. Coenzyme A is essential for enzymic acetylation of aromatic amines and for the acetylation of choline in brain (Lipmann, Kaplan, Novelli, Tuttle and Guirard, 1947). Pantothenic acid supplied to *Lactobacillus arabinosus* or *Proteus morganii* is converted into coenzyme A and raises the rate of pyruvate oxidation in pantothenate-starved cells. In yeast also, added pantothenate is converted into coenzyme A and the uptake of pantothenate is accompanied by a greatly increased rate of acetate metabolism (Novelli and Lipmann, 1947 *a* and *b*). There is no doubt, therefore, that coenzyme A has a wide significance in metabolism, but the detail remains to be filled in.

Added pantothenate is inactivated by various organisms during both aerobic and anaerobic carbohydrate metabolism (McIlwain and Hughes, 1944, 1945). Inactivation is related to metabolism, but not to growth, in a way reminiscent of the usage of nicotinamide and the phosphopyridine nucleotides during metabolism. This has been ascribed by Lwoff and by Morel to a " wearing out " of successive molecules of coenzyme in consequence of the continuous oxidation and reduction of its molecule (Lwoff and Lwoff, 1937*b*; Morel, 1941). Pantothenic acid has been implicated also as necessary for the synthesis of tryptophan by *Staph. aureus* (Sevag and Green, 1944*a*).

. . . . . .

Beside the essential metabolites to which definite enzymic or structural functions can be assigned, a number of other organic compounds have been found essential for animals and for growth of various nutritionally-exacting micro-organisms. The most important of these are biotin, *p*-aminobenzoic acid and folic acid. The functions of these substances have not yet

been correlated with any isolated enzyme systems, but it is probable that they will all be implicated as essential for various enzymic reactions.

Biotin

Biotin was originally recognised as a growth factor for yeast, but has subsequently been found to be essential for various clostridia, streptococci, staphylococci, pneumococci, lactobacilli and brucellæ. It is synthesised by other bacteria such as *Mycobacterium tuberculosis*, *Escherichia coli* and *Bacillus anthracis* (for full list see Knight, 1945; Peterson and Peterson, 1945).

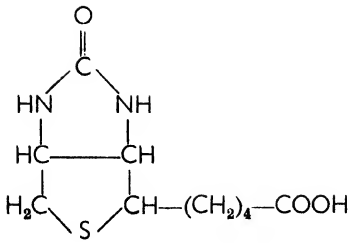
There is growing evidence for the metabolic function of biotin. du Vigneaud has suggested that it is involved in synthesis of some nitrogenous cellular material (Winzler, Burk and du Vigneaud, 1944). Biotin-deficient yeast cells were found to respire and ferment glucose at rates from one-tenth to one-twentieth of normal biotin-rich cells. The addition of biotin alone to the deficient cells had no effect, but biotin and ammonia caused a gradual rise in fermentation rate, followed by an increase in respiratory rate, and finally by an increase in growth rate. Biotin and ammonia were removed from the medium by deficient cells, but only if glucose was present; ammonia was not essential to biotin uptake. Azide, which is known to inhibit biosynthesis, prevented ammonia uptake at low concentrations.

Many lactic acid bacteria require both biotin and aspartic acid for growth and can be induced to grow without aspartic acid by increasing the biotin content of the medium (Stokes, Larsen and Gunness, 1947). Aspartic acid is not able to replace biotin for these organisms, so that biotin has probably some function other than promoting aspartic acid synthesis. The mechanism of the biotin-sparing action of aspartic acid is suggested by a report that oxaloacetic acid, a precursor of aspartic acid, also promoted growth of biotin-deficient lactobacilli. Synthesis of oxaloacetate by a number of heterotrophic organisms involves carbon dioxide fixation (see p. 77). Lardy, Potter and Elvehjem (1947) found that,

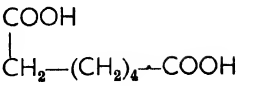
in a biotin-deficient medium, additional bicarbonate elicited no response, but that, in the presence of biotin, additional bicarbonate stimulated growth. The fixation of carbon dioxide in the form of oxaloacetate by carboxylation of pyruvate also seems to require biotin in yeast cells. In *Escherichia coli*, α-ketoglutaric acid or glutamic acid had a biotin-sparing effect when biotin synthesis was inhibited by imidazolidonecaproic acid (Fig. 15) (Shive and Rogers, 1947).

Degradation products and structural analogues of biotin promote growth in some micro-organisms and may have growth-inhibitory effects on others. The compounds to be discussed are depicted and numbered in Fig. 15.

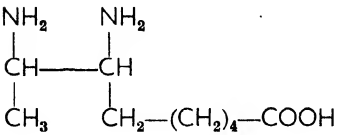
Pimelic acid (II) is a growth factor for some strains of *Corynebacterium diphtheriæ*, but can be replaced by lower concentrations of biotin. Such strains only lack the ability to synthesise the fatty acid, and if supplied with this can convert it to biotin. Desthiobiotin (V) had full biotin activity for some yeasts, but with the more exacting *Lactobacillus casei*, *Lactobacillus arabinosus* or *Staph. aureus* it not only failed to support growth but even acted as a growth inhibitor (Dittmer, Melville and du Vigneaud, 1944; Lilly and Leonian, 1944). *Neurospora* and exacting X-ray "mutants" of *Escherchia coli* or *Penicillium notatum* were able to use desthiobiotin in place of biotin; but a mutant of *Penicillium chrysogenum* was unable to utilise desthiobiotin, and synthesis and accumulation of desthiobiotin by this organism was demonstrated (Tatum, 1945*b*). Biotin diamino acid (IV) had some growth-promoting activity for yeast, and this was dependent on the carbon dioxide partial pressure, as might be expected if carbon dioxide acts as a source of carbon to complete the ring (Burk and Winzler, 1943). Diaminopelargonic acid (III) had about 10 per cent. of the activity of biotin for yeast, but was inactive for other organisms. Imidazolidonecaproic acid (VII) was growth-inhibitory to *L. casei* and to yeast, whereas the lower homologue, imidazolidonevaleric acid, was a poor growth factor for yeast and had no effect on *L. casei*. Biotin sulphone (VI) also possessed feeble growth-promoting activity for yeast, but was growth-inhibitory for *L. casei*



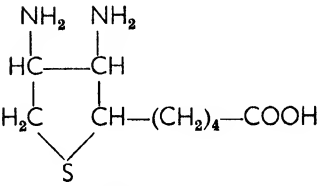
I. Biotin



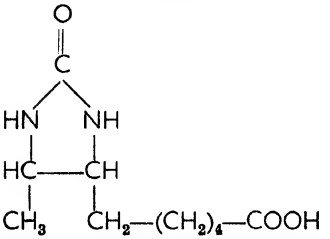
II. Pimelic acid



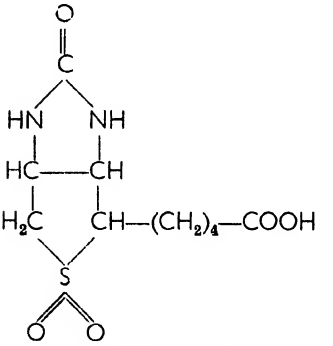
III. Diaminopelargonic acid



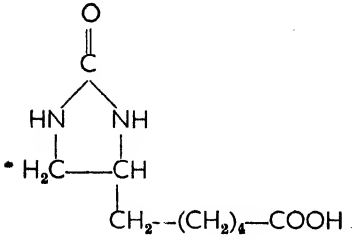
IV. Biotin diamino acid



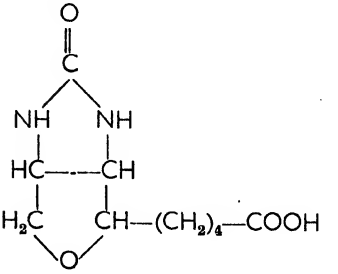
V. Desthiobiotin



VI. Biotin sulphone



VII. Imidazolidonecaproic acid



VIII. Oxybiotin

FIG. 15.—Biotin Analogues.

(Dittmer and du Vigneaud, 1944). Oxybiotin (VIII), the analogue in which the sulphur atom is replaced by oxygen, showed up to 50 per cent. of the growth-promoting activity of biotin for both yeast and *L. casei* (Pilgrim, Axelrod, Winnick and Hofmann, 1945; Rubin, Flower, Rosen and Drekter, 1945). Evidently oxybiotin can be utilised by yeast without preliminary conversion to biotin, since yeast grown on oxybiotin was found to contain oxybiotin in place of biotin (Hofmann and Winnick, 1945; Axelrod, Flinn and Hofmann, 1947).

These results demonstrate effectively the narrow distinction between essential metabolites and metabolite analogues; the latter may be classed as growth factors, growth-factor precursors, or growth inhibitors according to the metabolic capacities of the organisms studied. We shall return to these and related examples when discussing the design of chemotherapeutically-effective metabolite analogues in Chapter V.

p-*Aminobenzoic acid and folic acid*

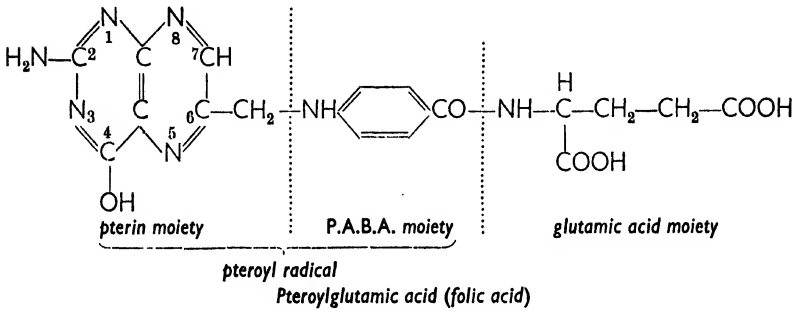
p-Aminobenzoic acid (P.A.B.A.) was found to be a growth factor only after Woods (1940) had shown that it acted as a powerful and specific antagonist to sulphanilamide (Chapter V). On the basis of this finding Woods suggested that P.A.B.A. was an essential metabolite.

The majority of micro-organisms are able to dispense with P.A.B.A. in their growth media; some of these have been found to synthesise their own supply (Landy, Larkum and Oswald, 1943). A few bacteria, including some strains of *Corynebacterium diphtheriæ*, *Clostridium acetobutylicum*, *Lactobacillus arabinosus* and *Acetobacter suboxidans*, have been found to require an external supply of P.A.B.A. (*cf.* Knight, 1945; Sarett, 1947).

One function of P.A.B.A. has been elucidated following the disclosure of the structure (see formula) of folic acid (pteroylglutamic acid) (Angier *et al.*, 1946; Mowat *et al.*, 1948). Various bacteria synthesise folic acid, and P.A.B.A. is required for this synthesis (Sarett, 1947).

Discovery of the wide distribution of folic acid and

recognition of its biological importance represented the confluence of many diverse researches on bacterial growth factors and on animal nutrition (review by Pfiffner and Hogan, 1946). Various forms of pteroylglutamic acid have been identified in which more than one molecule of glutamic acid is present; pteroyltriglutamate and pteroylheptaglutamate have been isolated from yeast (Pfiffner, Calkins, Bloom and O'Dell,



1946). These forms have been identified with one or other of the nutritional factors—vitamin B_c, vitamin B_c conjugate, yeast *L. casei* factor, liver *L. casei* factor, etc. The different-sized peptides show varying relative activities with micro-organisms, probably because some cells can degrade the larger peptides easily, whereas others are unable to do so. A carboxy-peptidase type of enzyme which can degrade pteroylheptaglutamate to pteroylmonoglutamate has been identified in animals and in yeast (*cf.* Hutchings *et al.*, 1948).

Some micro-organisms require folic acid and are unable to synthesise it for themselves (Daniel, Norris, Scott and Heuser, 1947), others synthesise their own requirements. P.A.B.A. is an essential growth factor for *L. arabinosus*. Growth of *L. arabinosus* in the presence of P.A.B.A. is accompanied by the synthesis of pteroylglutamic acid, but pteroylglutamic acid can replace P.A.B.A. only to a very limited extent (Sarett, 1947). This suggests that P.A.B.A. has other functions in the organism besides the synthesis of pteroylglutamic acid.

With an essential metabolite of the molecular complexity of folic acid, the full detail of its structural specificity will

take some time to work out, but it is already apparent that some pteroylglutamic acid analogues possess limited growth-promoting properties and that others are growth inhibitory. N-(4-(4-quinazoline)-aminobenzoyl)-glutamic acid has from one-tenth to one-hundredth of the activity of folic acid for *Lactobacillus casei* (Martin, Moss and Avakian, 1947). Several 2 : 4-diaminopterins (for numbering see formula) inhibit growth of *Strep. fæcalis* and *L. casei* which require preformed folic acid, and also *L. arabinosus*, *Escherichia coli* and *Staph. aureus* which synthesise their own supply (Daniel, Norris, Scott and Heuser, 1947 ; Daniel and Norris, 1947). A full discussion of the relationship between P.A.B.A., folic acid, sulphonamides and other inhibitors of folic acid synthesis is deferred until Chapter V. There is considerable evidence of a relationship between folic acid, purine and pyrimidine synthesis, but more information will have to be collected before the position becomes clear (see pp. 203-213). One hint of a possible coenzyme function for folic acid is provided by the report that 7-methylfolic acid acts as an inhibitor of " dopa " decarboxylase and that inhibition is reversed by folic acid (Martin and Beiler, 1947).

Conclusion

Substances probably act as growth inhibitors because they are in one way or another enzyme inhibitors. The classification of a compound as an essential metabolite, is equivalent to saying that it is a constituent part of some enzymic system within the cell, and that enzymes must exist which are specifically designed to " fit " the particular chemical arrangement characteristic of the metabolite. If the enzyme is presented with a compound of closely related structure which can " fit " it the same way, but which is nevertheless in some other respect unusable for the functions normally performed by the metabolite, then the metabolite analogue may act as a growth inhibitor. The most important groups of metabolite analogues from the chemotherapeutic point of view are discussed more conveniently after a general review of the

characteristics of enzyme inhibition and of the possible ways in which various types of enzyme inhibition may differ.

It should be kept in mind that not only coenzyme or prosthetic group analogues may function in this way. Any molecule whether organic or inorganic, may resemble a functional metabolite in such a way that it is caught up in the overall catabolism and anabolism of the living cell, and may form in some metabolic chain a non-functional break which will upset the finely balanced processes of life and lead to death or to failure of reproduction.

We have on several occasions drawn attention to the capacity of an organism to dispense with what are apparently essential growth factors when placed in different media. This capacity should be kept in mind when considering the mode of action of chemotherapeutic drugs. Pathogenic organisms in their natural state, as parasites on the tissues of a host, can draw upon a multitude of nutrilites, and the growth-inhibitory action of a drug may be overcome by a shift in metabolic balance which eliminates the strain's need for the drug-sensitive enzyme system. Such an altered balance may not be possible in a limited synthetic medium so that a drug effective *in vitro* may be useless *in vivo*, or rapidly rendered useless by the development of drug resistance.

CHAPTER IV

ENZYME INHIBITION

We have attempted to build up a composite, but admittedly incomplete, picture of intermediary metabolism of living cells, particularly of bacterial cells as shown by their nutritive requirements. Examination of this picture may help in elucidating the mode of action of known chemotherapeutic drugs and in developing new ones. First, it is essential to deal briefly with the kinetics of enzymic reactions and also to study more fully the subject of enzyme inhibition, so that we can distinguish between the various types of inhibition known to occur.

Enzyme kinetics

The velocity of enzyme-catalysed reactions follows certain general rules which may be applied to most enzymes. At controlled *p*H and temperature, the velocity is constant during the early part of the reaction, but subsequently declines progressively with time due to exhaustion of substrate, accumulation of end-products and other causes. Unless otherwise stated, the velocity of enzymic reactions is always measured during this initial period when it is constant.

Each enzyme has a certain optimal *p*H at which it reacts most rapidly with a particular substrate. There is also usually an optimal temperature for each enzyme, above which heat-inactivation of the enzyme plays a part in determining the reaction velocity. With low substrate concentrations, velocity is proportional to both enzyme and substrate concentration, but with increasing substrate concentration a maximal velocity is reached which is no longer dependent on substrate concentration, being proportional only to enzyme concentration (Fig. 16). Enzymic reactions are reversible; the effect of enzymes, like all catalysts, is to speed the attainment of equilibrium but not to alter the position of equilibrium.

Theoretically, enzymic reactions should conform to the law of mass action, with velocity proportional only to substrate concentration. We have already seen that this is not the case, and Fig. 16 shows a comparison between the velocities of two reactions, one enzymic and one following the law of mass action. This discrepancy was first explained satis-

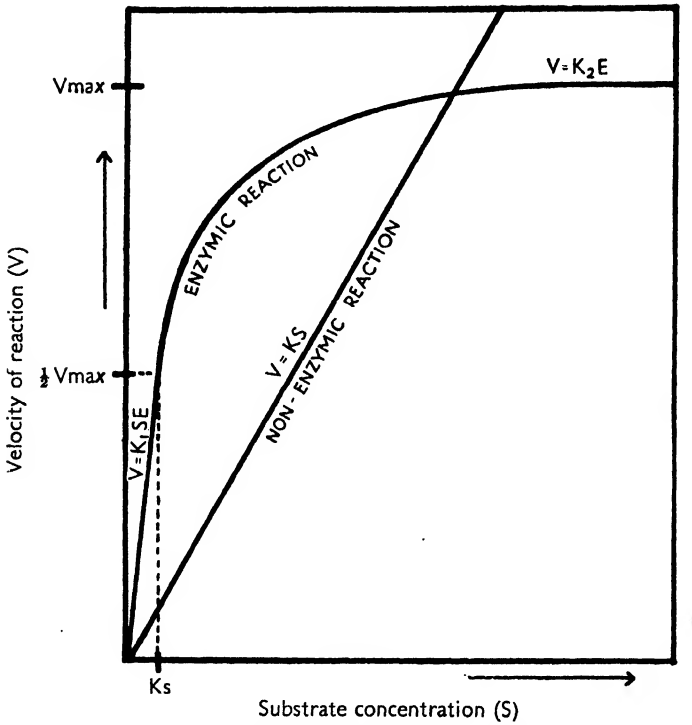


FIG. 16.—Effect of substrate concentration on reaction velocity; enzymic and non-enzymic reactions.

factorily by Michaelis and Menten (1913), who assumed that enzyme and substrate combine reversibly to form an intermediate complex. Applying the law of mass action to this reversible reaction, they obtained an equation, known as the Michaelis-Menten equation, for variation of speed of reaction with substrate and enzyme concentrations. This equation provides both adequate explanation of much experimental

data, and useful constants for the characterisation of individual enzymes.

The derivation of the Michaelis-Menten equation can be explained by reference to the enzyme saccharase as an example ; this catalyses the following overall reaction :—

$$\begin{matrix}\text{Sucrose} \\ + \\ H_2O\end{matrix} \xrightleftharpoons{\text{SACCHARASE}} \begin{matrix}\text{Glucose} \\ + \\ \text{Fructose}\end{matrix}$$

According to the Michaelis theory, the reaction proceeds in two stages : (*a*) one molecule of sucrose and one of water combine with one molecule of enzyme to form a complex in equilibrium, according to the law of mass action, with its constituents ;

$$\boxed{\text{SACCHARASE}} + \begin{matrix}\text{Sucrose} \\ + \\ H_2O\end{matrix} \rightleftharpoons \boxed{\text{SACCHARASE} \begin{matrix}\text{Sucrose} \\ H_2O\end{matrix}} \quad -(a)$$

(*b*) the intermediate complex splits to form the reaction products glucose and fructose, and unchanged enzyme. The assumption

$$\boxed{\text{SACCHARASE} \begin{matrix}\text{Sucrose} \\ H_2O\end{matrix}} \rightleftharpoons \boxed{\text{SACCHARASE}} + \begin{matrix}\text{Glucose} \\ + \\ \text{Fructose}\end{matrix} \quad -(b)$$

is made that the rate of reaction, at any time, is governed by the concentration of the enzyme-substrate complex, since the rate of formation of this complex (reaction *a*) is very much more rapid than that of its decomposition into reaction products (reaction *b*). Thus the concentration of complex may be obtained by applying the law of mass action to reaction (*a*) alone.

The reaction proceeds in aqueous solution, thus the concentration of water is infinite in comparison to that of the other reactants and can be neglected.

Reaction (*a*) may therefore be represented as

$$\begin{matrix}\text{Sucrose} \\ \text{S}\end{matrix} + \begin{matrix}\text{Enzyme} \\ (e-p)\end{matrix} \rightleftharpoons \begin{matrix}\text{Enzyme} - \text{Sucrose} \\ p\end{matrix}$$

Let S = concentration of sucrose
e = concentration of total enzyme
p = concentration of enzyme-sucrose complex
$e-p$ = concentration of free enzyme.

By the law of mass action, the velocity (v_1) of formation of enzyme-sucrose complex is proportional to the product of the concentration of reactants, *i.e.* $v_1 = K_1S(e-p)$ where K_1 is a constant. At equilibrium, this is equal to the rate v_2 of the reverse reaction, where $v_2 = K_2p$,

i.e. $v_1 = v_2$ and $K_1(e-p)S = K_2p$

$$\therefore \quad eS - pS = \frac{K_2}{K_1}p$$

$$= K_sp, \text{ where } K_s = \frac{K_2}{K_1}$$

Whence $p(K_s+S) = eS$

and
$$p = \frac{eS}{K_s+S} \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

Now p is the concentration of enzyme-substrate complex, which is assumed to govern the overall velocity V of the hydrolytic reaction.

In other words, $V = cp$ (where c = constant) . . (2)

Substituting for p in equation (1)

$$V = \frac{ceS}{K_s+S} \quad . \quad . \quad . \quad . \quad (3)$$

$$= \frac{ce}{\frac{K_s}{S}+1}$$

Then V approaches a maximum value, V_{max}, as S approaches infinity and $V_{max} = ce$.

Whence, substituting for V_{max} in (3)

$$V = \frac{V_{max}S}{K_s+S} \quad . \quad . \quad . \quad . \quad (4)$$

This is the original form of the Michaelis-Menten equation ; it can also be written as

$$K_s = S\left(\frac{V_{max}}{V}-1\right) \quad . \quad . \quad . \quad (5)$$

(For less simplified derivation of this equation, see Hellerman, Lindsay and Bovarnick, 1946).

K_s (the dissociation constant of the enzyme-substrate complex) is known as the *Michaelis constant*; it represents an important constant which is independent of enzyme and substrate concentration and is characteristic of each enzyme. K_s may be considered to represent a measure of the affinity of an enzyme for its substrate. When $V = \frac{1}{2}V_{max}$, $K_s = S$; that is, K_s is numerically equal to substrate concentration when the velocity is half the maximum value (see Fig. 16).

An accurate knowledge of K_s and V_{max} are essential in characterising an enzyme. They could, of course, be obtained from equation (5) by plotting velocity against substrate concentration as in Fig. 16. A less laborious and more accurate method suggested by Lineweaver and Burke (1934) is to plot the reciprocals of velocity and substrate concentration and so obtain a straight line whose equation (6) is derived from equation (4).

$$\frac{1}{V} = \frac{K_s + S}{V_{max}\,S}$$

i.e.

$$\frac{1}{V} = \frac{K_s}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}} \quad . \quad . \quad . \quad (6)$$

The slope of this line is $\frac{K_s}{V_{max}}$, while its intercept on the $\frac{1}{V}$ axis is equal to $\frac{1}{V_{max}}$ (see Fig. 17).

The above theory and the formulæ derived from its application have proved adequate to cover the kinetic data for many enzymic reactions; Stearn (1938) concludes that the Michaelis constant may possess real thermodynamic significance. However, as might be expected from the enormous variety of enzymic reactions, not every case will fit into the mould prepared by Michaelis and Menten. Various explanations have been put forward to explain these anomalies (Wilson, 1939). Briggs and Haldane (1925) pointed out that the assumption is not always justified that formation of the intermediate enzyme-substrate complex is infinitely rapid compared to its rate of decomposition into enzyme and reaction products; in certain cases rate of formation of the complex might be limited by the number of collisions in the

bi-molecular reaction Enzyme+Substrate→Complex. The application of chain reaction theory has led to an expression derived by Moelwyn-Hughes (1933, 1937, 1940) for velocity of reaction; Medwedew (1937) replaced the intermediate

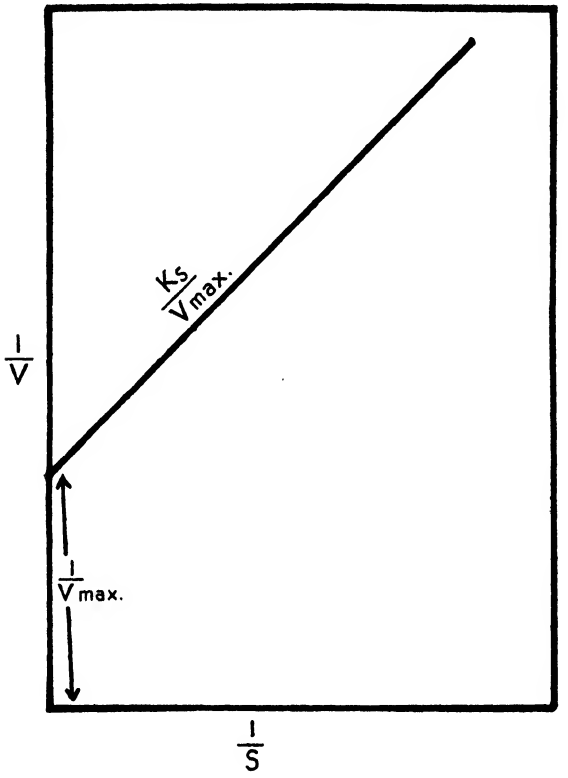


FIG. 17.—Variation of reciprocal of velocity (1/V) of enzymic reaction with reciprocal of substrate concentration (1/S). (Enables V_{max}, maximum velocity, and K_s, dissociation constant of enzyme-substrate complex, to be calculated graphically.)

complex theory by one based on quantum mechanics in which there is no formation of a definite complex between enzyme and substrate; Stearn (1938) applied quantum mechanics to enzyme-substrate complex. Each of these theories explains some anomaly in the kinetics of certain enzyme reactions, but they all (including that of Michaelis and Menten) predict

equally satisfactorily the same set of experimental data (*e.g.* Fig. 16) derived from the more common enzymic reactions. Therefore one must conclude that, in this field, agreement between experimental and predicted data does not constitute proof of a theory. For most practical purposes, the Michaelis-Menten theory and equations may be used to derive characteristic enzyme constants, and as we shall see later, also provide explanations of the phenomena of enzyme inhibition.

Enzyme-substrate complex

The question of the reality of existence of an enzyme-substrate complex is of importance in connection with the phenomena of enzyme inhibition as well as in the derivation

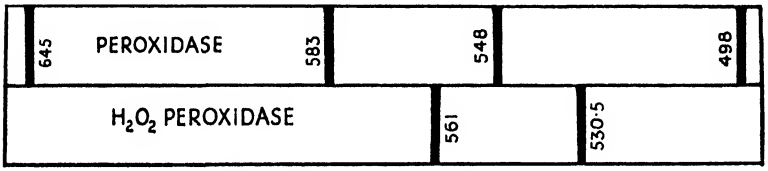


FIG. 18.—Positions of absorption bands of peroxidase and H_2O_2-peroxidase compound. (Keilin and Mann, 1937.)

of the velocity constants of enzymic reactions. Direct evidence has been difficult to obtain because of the extremely short life of the complex, but work with peroxidase has shown that, in the case of this enzyme at any rate, a definite complex is formed (Keilin and Mann, 1937). Peroxidase is a hæmatin-containing enzyme which decomposes hydrogen peroxide in the presence of an oxidisable compound to which it transfers one atom of oxygen. Its spectrum conforms to that of an iron-porphyrin compound in the ferric state with absorption bands at 645 mμ, 583 mμ, 548 mμ, and 498 mμ. On addition of hydrogen peroxide to a strong solution of peroxidase, the original brown colour of the solution is changed to a clear red and the four-banded absorption spectrum is replaced by two bands at 561 and 530·5 mμ (Fig. 18). The amount of peroxide necessary to effect such a change corresponds to one molecule per atom of porphyrin iron. In the absence of an oxygen

acceptor the two-banded form of peroxidase is fairly stable, but on addition of pyrogallol the four-banded spectrum immediately reappears. The evidence suggests that enzyme and substrate are here combining to form a compound whose spectrum differs from that of the free enzyme.

This work has been extended by Chance (1943) using leuco-malachite green as an oxygen acceptor. A photo-electric

$$\left.\begin{matrix} H_2O_2 \\ + \\ \text{Leuco-malachite green} \end{matrix}\right\} \xrightarrow{\text{PEROXIDASE}} \left\{\begin{matrix} 2H_2O \\ + \\ \text{Malachite green} \end{matrix}\right.$$

recording device measuring rapid changes in absorption at suitable wave lengths enabled the velocity of formation and dissociation of the peroxidase-H_2O_2 complex to be determined. These velocities were found to follow bi-molecular reaction laws; complex formation was extremely rapid, while the reverse reaction was relatively slow. The rate-determining step of the overall reaction was a much slower monomolecular decomposition of the complex into enzyme and reaction products. These experiments provide proof of the two main assumptions on which Michaelis based his theory, namely formation of enzyme-substrate complex, and the dependence of rate of reaction on speed of decomposition, not of formation, of this complex. It would appear then that for our purpose, the Michaelis treatment of enzyme kinetics is adequate.

The prosthetic groups of some enzymes are sufficiently firmly bound to the protein portion of the molecule to be regarded as part of the complete enzyme as in the case of peroxidase. In other enzymes, such as the pyridine nucleotide dehydrogenases, the prosthetic group is easily dissociated and is usually referred to as a coenzyme. In such cases, the coenzyme can be regarded as a second substrate undergoing reversible combination with the protein molecule, and its reactions are therefore subject to the same kinetic treatment as the enzyme-substrate complex itself.

Non-specific enzyme inhibition; "SH inhibition"

Enzymes are generally assumed to combine with their substrates by means of their "active centres" or "active

groups." Little is known about the nature of these active centres, but it is likely that they are responsible for the highly specific effect of each enzyme. For the purpose of visualising the combination of enzyme with its substrate, the lock and key theory propounded by Fischer (1894) is still of value, since it emphasises that the configuration of both enzyme and substrate must conform to a certain pattern. This is well illustrated by the work of Bergmann and his school on proteolytic enzymes and the optical configuration of their substrates. Certain of these enzymes, such as carboxypeptidase, are incapable of hydrolysing synthetic peptides whose terminal amino acids are of the *d*-configuration, although the *l*-antipodes are extremely sensitive to action of the enzyme (Stahmann, Fruton and Bergmann, 1946).

It is reasonable to conclude that any substance which reacts with an essential group at the active centre of the enzyme, will inhibit by rendering the enzyme incapable of combining with or activating its substrate. As we shall see, this inhibitory effect is not confined to substances reacting only with the active centre, and it is probable that certain other groupings in the enzyme are also essential for maintenance of activity. Langenbeck (1935), from a study of the catalytic properties of certain synthetic organic compounds, suggested that a distinction should be drawn between the active centre at which substrates react and the surrounding groups in the molecule which may have an activating effect on the active centre. Such a concept is also useful in the case of enzymic catalysis, and should be borne in mind in the ensuing description of enzyme inhibition.

Any substance which causes irreversible denaturation of proteins in general will act as a non-specific enzyme poison, and is of no interest either for theoretical consideration of enzyme inhibition or as a chemotherapeutic drug. On the other hand, selective reagents which react with free carboxyl, hydroxyl, amino and sulphydryl groups of proteins should yield valuable information as to the nature of essential groups, provided pure enzymes are employed. Careful choice of reagent may result in blocking or alteration of one or more of certain

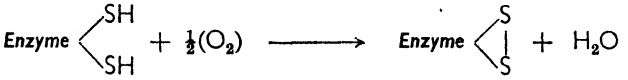
reactive groups of an enzyme without affecting other groups. For example, the use of nitrous acid and ketene, which react with amino and aromatic hydroxy groups, has shown that free amino groups are not essential for activity of β-amylase, pepsin or chymotrypsin, but that free tyrosine hydroxy groups are essential (Weill and Caldwell, 1945; Philpot and Small, 1938; Herriott and Northrop, 1934; Herriott, 1935, 1936; Sizer, 1945). Free amino groups have, however, been found necessary for activity of pancreatic amylase and alkaline phosphatase (Little and Caldwell, 1943; Gould, 1944). Iodine has also been used to demonstrate the essential nature of free tyrosine hydroxyl radicals in pepsin; Li (1945) found that out of the total 17 tyrosine residues present in the molecule, only 12 were free to react with iodine.

Selective attack or blocking has yielded the most fruitful results in the case of sulphydryl groups, which have been thus found essential for activity of many enzymes. Sulphydryl groups are highly reactive, combining with a variety of reagents, and are also susceptible to oxidation by mild oxidising agents. This reactivity is illustrated by the case of the amino acid cysteine, which is a main source of sulphydryl groups in proteins. In air, solutions of cysteine are rapidly oxidised in the presence of catalytic amounts of metals, but are stable in the complete absence of metals. The product of oxidation is cystine, the corresponding disulphide derivative; reducing agents reverse the reaction, and free sulphydryl groups are regenerated.

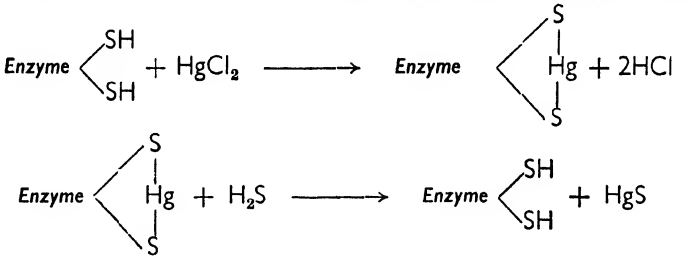
$$\underset{\text{Cysteine}}{2[SH.CH_2.CH(NH_2).COOH]} + \tfrac{1}{2}(O_2) \xrightarrow{\text{(metal catalysis)}} \underset{\text{Cystine}}{COOH.CH(NH_2).CH_2.S{-}S.CH_2.CH(NH_2).COOH} + H_2O$$

Many enzymes have been known for some time to be susceptible to the action of mild oxidising and reducing agents (see review by Hellerman, 1937), but the earlier reports related only to crude enzyme preparations and led to no acceptable theory for the mechanism of this effect. However, in 1933, Hellerman observed that highly purified crystalline

urease was extremely easily inactivated in air by catalytic traces of heavy metals in the solution ; iodine-iodide mixtures had a similar effect. Both inactivations could be reversed by reducing agents. Working by analogy with cysteine, he suggested that these inactivations of urease were due to oxidation of sulphydryl groups essential for enzyme activity (Hellerman, Perkins and Clark, 1933). The change may be represented as follows :—



This suggestion explained earlier observations of numerous investigators on the inactivation of urease by salts or ions of heavy metals, since the reactive sulphydryl groups could combine readily with metallic ions to form mercaptide linkages. This inactivation might be reversed by an excess



of some substance capable of forming a less dissociable or an insoluble compound with the metal. Such conditions occur in the long-known reactivation of urease by cyanide or hydrogen sulphide. The activating effect of reducing agents on the plant proteolytic enzyme papain was also explained in 1933 by Bersin as a reduction of —S—S-linkages to the sulphydryl form. In confirmation of this, he found that papain was inhibited by trivalent organic arsenical compounds, the inhibition being reversed by glutathione (Bersin, 1933 ; Bersin and Logemann, 1933).

Since this preliminary work, the activity of numerous enzymes has been shown by many investigators to be dependent on free sulphydryl groups, and the term "*SH enzymes*" is now widely applied to any such enzyme (see Barron, 1943, and

Barron and Singer, 1945 *a* and *b*, for list of such enzymes). Many reagents are employed to detect essential sulphydryl groups, among which, the most active and specific are the organic mercaptide-forming reagents such as *p*-chloromercuribenzoate (introduced by Hellerman) and the trivalent arsenoxides. Mild oxidising agents like porphyrindin, ferricyanide and *o*-iodosobenzoate, when acting under carefully controlled conditions, may also be used to attack sulphydryl groups specifically. The inactivation caused by both mercaptide-forming reagents and oxidising agents is reversed by low molecular weight thiols such as cysteine or glutathione ; this reversal should always be included as a further diagnostic test for " SH enzymes." Alkylating agents such as iodoacetate, iodoacetamide and bromoacetate were frequently used by earlier investigators in tests for " SH enzymes." Iodoacetate was shown by Dickens (1933) to react stoichiometrically with sulphydryl-containing compounds such as glutathione, and it is now generally believed to react with sulphydryl groups of proteins by alkylation. This reaction is, however, irreversible and not entirely specific to sulphydryl groups.

$$\text{Enzyme—SH} + \text{ICH}_2\text{COOH} \longrightarrow \text{Enzyme—S—CH}_2\text{COOH} + \text{HI}$$

Not all enzymes now known to require sulphydryl groups for activity are equally easily inactivated by iodoacetate or oxidising agents. Thus, iodoacetate at low concentrations (M/3000) is known to be an extremely potent inhibitor of yeast fermentation by reason of a specific inhibition of alcohol dehydrogenase (Dixon, 1937). Many other enzymes concerned in alcoholic fermentation are " SH enzymes " (see Barron, 1943), but none are inhibited by this concentration of iodoacetate. This must be due to differences in reactivity of sulphydryl groups caused by the configurations of the different enzymic proteins. This variation in reactivity is illustrated in the case of papain and urease. Papain is inactivated by iodoacetate, yet only one out of the total of 10 sulphydryl groups is alkylated by this reagent (Balls and Lineweaver, 1939). On the other hand, when crystalline urease was treated with one mole of *p*-chloromercuribenzoate per 21,300 g.

of enzyme no loss in activity occurred, but a second mole of reagent produced loss in activity, although only 2 out of a total of 5 sulphydryl groups were then combined; the remaining 3 sulphydryl groups could only be attacked after denaturation of the protein (Hellerman, Chinard and Deitz, 1943). Urease is not inactivated by porphyrindin, yet once oxidised by this reagent, it only requires one mole of *p*-chloromercuribenzoate for inactivation. These experiments suggest that urease contains 3 types of sulphydryl groups: a freely reactive one which is not essential for activity, another which is less reactive but essential for activity, while the third type is not free to react in the native protein and is only uncovered during the unfolding of the protein chains, which accompanies denaturation. Great differences occur in the susceptibility of various SH-enzymes to arsenical compounds. The war gas lewisite, $Cl.CH{=}CH.AsCl_2$, was found to be highly toxic for most SH-enzymes tested; organic trivalent arsenical drugs were less toxic, and arsenites were least effective. Three SH-enzymes were, however, almost unaffected by lewisite (Barron *et al.*, 1947).

The actual role played by sulphydryl groups in enzymes is still unknown; in several cases, however, there is evidence that they do occur at the active centre of the enzyme. Thus, succinic dehydrogenase is not inhibited by "SH inhibitors" if succinic or malonic acid is already present (Hopkins, Morgan and Lutwak-Mann, 1938; Potter and DuBois, 1943; Barron and Singer, 1945*a*). Phosphoglyceraldehyde dehydrogenase is protected to some extent by its coenzyme (diphosphopyridine nucleotide) from inhibition by "SH inhibitors" (Rapkine, 1938), while the inhibition of *d*-amino acid oxidase by *p*-chloromercuribenzoate is partly prevented by the presence of excess of its coenzyme (flavin adenine dinucleotide) (Hellerman, Lindsay and Bovarnick, 1946). In these cases, combination of the enzyme with either coenzyme, substrate, or substrate-analogue evidently reduced access of inhibitor to sulphydryl groups, and it is probable that here sulphydryl groups are playing an essential part in the formation of enzyme-substrate complex.

Whatever the role played by sulphydryl groups, it is evidently an important one for maintenance of enzymes in an active state. Glutathione can effect complete reversal of inactivation by *p*-chloromercuribenzoate only if it is added within ten to fifteen minutes after addition of the mercurial to the enzyme. After this time, reversal is not complete, the degree of reversal being decreased with increasing time interval between addition of inhibitor and antidote (Barron and Singer, 1945*a*).

With the realisation of the importance and widespread nature of enzymes depending for activity on sulphydryl groups, it is reasonable to assume that any substance capable of reacting with such groups may be a potential enzyme inhibitor. Conversely, any substance with chemotherapeutic activity possessing the capacity to link with reactive hydrogen may act by addition to certain sulphydryl groups of enzymes. Metal oxides or metal ions, quinones, ketones, acids or nitriles with $\alpha : \beta$-unsaturation might be expected to act in this way. Their selective action, whereby some "SH enzymes" are inhibited by certain reagents and others are not, is probably due to differences in accessibility of the essential sulphydryl groups of various enzymes.

Specific inhibition; competitive and non-competitive

So far we have dealt with types of inhibition which are relatively non-specific in that they are concerned with certain groupings common to many enzyme proteins. If an inhibiting agent proves to be specific to one enzyme or group of enzymes, it may reasonably be assumed to interfere with a reaction or grouping specific to those enzymes. Enzymic specificity is related to the active centre, which is so constituted as to combine selectively with the substrate. Therefore, any substance which prevents either this combination or the activation of substrate on the enzyme surface may act as a specific inhibitor to the enzyme in question. We may envisage several ways by which such inhibition could be brought about. Access of either coenzyme or substrate to the active centre may be prevented by alteration or blocking of the centre

itself, or the coenzyme or the prosthetic group may be modified so that it is incapable of performing its function.

Alteration of prosthetic group is responsible for inhibition of the hæmatin enzymes, such as cytochrome oxidase and catalase, by carbon monoxide, hydrogen sulphide, cyanide, azide or hydroxylamine. The inhibition of glycolysis by fluoride is now known to be due to reaction with magnesium, a coenzyme of enolase (Warburg and Christian, 1942). Fluoride had long been known to be a powerful specific inhibitor of glycolysis, causing accumulation of phosphoglyceric acid (Emden and Ickes, 1934). When Warburg crystallised the enzyme enolase which acts on phosphoglyceric acid, he found, as expected, that it was strongly and specifically inhibited by fluoride. The mechanism of inhibition was shown to be through formation of a magnesium-fluoro-phosphate complex which inhibits the action of the enzyme by itself combining with enzyme protein. The inhibition of certain metalloprotein enzymes by 2 : 3-dimercaptopropanol (B.A.L.) and other dithiols is also due to combination of metal with inhibitor (Webb and van Heyningen, 1947 ; Barron, Miller and Meyer, 1947).

In many cases the specificity of an enzyme for its substrate or coenzyme is not absolute, and the enzyme is capable of combining through its active group with other substances structurally related to either substrate or coenzyme. However, it is unable to activate these substances and is consequently inhibited because access to the active group is prevented. In other words, a competition for the active group exists between inhibitor and substrate or coenzyme. The term *competitive inhibition* is therefore applied to the phenomenon, which is of considerable importance in enzyme chemistry. Malonic acid, the lower homologue of succinic acid, inhibits succinic dehydrogenase by preventing access of succinic acid to the enzyme. This can be shown by increasing the ratio of succinic acid to malonic acid in the reaction system ; the degree of inhibition is then decreased, owing to displacement by succinic acid of some of the malonic acid in combination with the enzyme. With sufficient succinic acid, the inhibition can be completely overcome (Hopkins, Morgan

and Lutwak-Mann, 1938). Combination of inhibitor with enzyme is easily reversible, and the degree of inhibition for a given amount of enzyme is related to the concentration of substrate as well as to the inhibitor concentration. These are characteristic properties of true competitive inhibition and are used to identify the phenomenon both *in vitro* and *in vivo*. Malonic acid acts as a very specific inhibitor *in vivo* of succinic dehydrogenase; its use in this respect has already been referred to in considering the tricarboxylic acid cycle (p. 80). Some other substances related to succinic acid which have been found to inhibit succinic dehydrogenase are

$COOH.(CH_2)_3.COOH$ $C_6H_5.CH_2.CH_2.COOH$

$COOH.CH(C_2H_5).COOH$ $C_6H_5.CH_2.COOH$

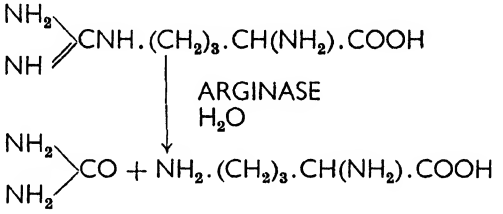
$$COOH.CH_2.CH(COOH).CH_2.COOH$$

$C_6H_5.CH(OH).COOH$

(Quastel and Wooldridge, 1928). Many other cases of competitive inhibition are known, among which may be cited the inhibition of diamine oxidase by certain long-chain diamines (Blaschko and Duthie, 1945*b*); the inhibition by glucose of the phosphorylase synthesising glycogen from glucose-6-phosphate (Cori, Cori and Green, 1943); and the inhibition by pyridine sulphonic acid of glucose and lactic acid dehydrogenases both of which work through coenzyme I (Euler, 1942). In the latter case, the coenzyme is displaced from combination with enzyme protein by the sulphonic acid analogue of the coenzyme; in the two former cases, it is the substrate which is in competition with inhibitor.

Another type of specific and reversible inhibition is frequently encountered, in which degree of inhibition is not related to substrate concentration, but only to inhibitor concentration. This suggests that the substrate cannot prevent combination of inhibitor with enzyme. Therefore, the inhibitor must combine with some grouping which does not itself combine with the substrate but which is essential for

substrate activation. This type of inhibition is usually referred to as *non-competitive*, to distinguish it from competitive inhibition. Like competitive inhibition, it is also displayed by substrate analogues, but when both competitive and non-competitive inhibition of an enzyme occur with different inhibitors, those analogues giving rise to competitive inhibition usually resemble the substrate more closely than those causing non-competitive inhibition. Such is the case in the inhibition of arginase by amino acids, studied by Hunter and Downs (1945). This enzyme catalyses the splitting of arginine to urea and ornithine. The diamino acid lysine inhibits the action



of arginase in a competitive manner, the degree of inhibition for a given enzyme concentration being dependent on inhibitor/arginine ratio. Monoamino acids, on the other hand, show non-competitive inhibition, where substrate concentration has negligible effect on the extent of inhibition. Fig. 19 shows graphically the effect of amino acids on arginase, residual enzyme activity times inhibitor concentration being plotted against arginine concentration (see p. 167 for derivation of relation).

In Fig. 19 we can see that ornithine, one of the reaction products, inhibits arginine breakdown in an apparently competitive manner. Consideration of the equilibrium of this reaction shows that either of the reaction products could act in this way by preventing dissociation of the enzyme-arginine complex, the extent of breakdown being obviously proportional to the concentrations of the various reactants. The inhibition of enzymic reactions by excess of one reaction-product is a fairly general effect. It may be encountered, for example, in bacterial suspensions to which a large excess of

some metabolite has been added. It is often misinterpreted as indicating that the metabolite is inhibitory *per se*, and erroneous theories have sometimes been based on such observations.

The foregoing account of enzyme inhibition has tended to over-simplify the phenomenon for purposes of classification. The divisions between specific and non-specific inhibitors and

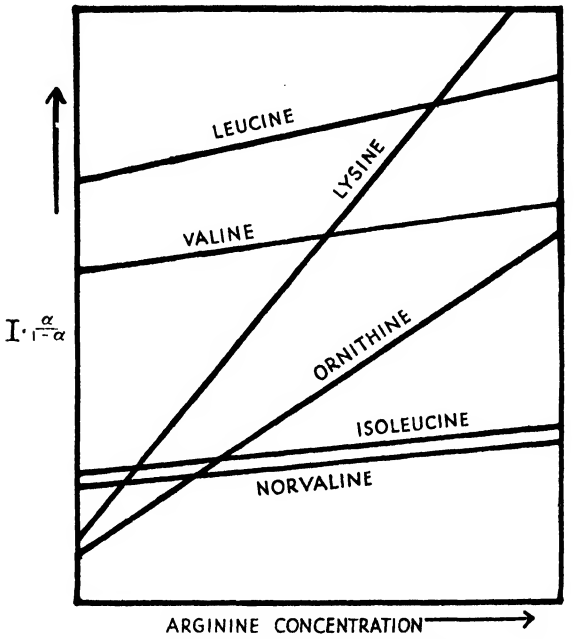


FIG. 19.—Inhibition of arginase by *l*-amino acids. I = concentration of inhibiting amino acid. α = fractional activity, where $1-\alpha$ = degree of inhibition. (Hunter and Downs, 1945.)

competitive and non-competitive are only valid when applied to one particular enzyme. An inhibitor may combine reversibly with a group at the active centre of one enzyme and show the characteristics of competitive inhibition, while with another enzyme it may attack a chemically similar group situated outside the active centre and will be classed as a non-competitive inhibitor. This would occur if the particular grouping which combined with the inhibitor was essential in

both cases but was concerned in combination with substrate or coenzyme only in the first case. The work of Hellerman and his co-workers on atebrin and quinoline bases as enzyme inhibitors suggests that all these compounds combine with similar groups in different enzymes, the resulting inhibition varying in type with the enzyme in question (Hellerman, Lindsay and Bovarnick, 1946). Any enzyme inhibited by one of these compounds was found to be inhibited by the whole group, the order of effectiveness in the group being similar for different enzymes. The enzymes *d*-amino acid oxidase, diaphorase, lactic dehydrogenase, pancreatic lipase and catalase were all inhibited by atebrin, plasmoquine, quinine and various non-antimalarial quinolines. The inhibition of *d*-amino acid oxidase by quinine was found to be a true competitive inhibition with respect to the coenzyme, flavin adenine dinucleotide ; while with diaphorase, non-competitive inhibition was reported. Atebrin showed true competitive inhibition with pancreatic lipase, but its effect on *d*-amino acid oxidase was found to be not strictly competitive when investigated kinetically.

Inhibition by atebrin of flavoproteins, when first reported, was thought to be due possibly to a structural similarity between the atebrin and flavin molecules, since protection from inhibition could be afforded by the flavin-containing coenzyme (Wright and Sabine, 1944 ; Haas, 1944). However, the findings by Haas that atebrin also inhibited glucose-6-phosphate dehydrogenase, whose pyridine-nucleotide coenzyme could also protect against inhibition, suggested that this could not be the case. Quinine was also found to inhibit both these enzymes. Extension of the investigation by Hellerman and co-workers showed that these coenzyme antagonisms are not specific to atebrin, but are also shown by numerous quinolines, and cannot therefore be due to structural similarity with a portion of the coenzyme molecule. This was further stressed by the fact that all these compounds inhibit other totally unrelated enzymes. The one common property shown by all these inhibitors and the coenzymes is their basic nature. It is possible that they combine with certain acidic groups of the enzymes, and where such groups are concerned with

combination with coenzyme, a competition between coenzyme and inhibitor will occur.

We can see then that competitive inhibition is not necessarily confined to substrate analogues, and may not even be specific to one enzyme or class of enzymes. The danger of misinterpreting the action of an inhibitor as a result of testing on an insufficient number of enzymes is well illustrated in the case of the antimalarial drugs.

Kinetics of inhibition

A knowledge of the dissociation constant of enzyme-inhibitor complex is of great importance in assessing the potency of an inhibitor and the possibility of employing it *in vivo*. A study of the kinetics of enzyme inhibition provides a means for determining both this constant and, at the same time, the equally important dissociation constant of the enzyme substrate complex (Michaelis constant). It also provides mathematical proof, should it be required, for the differences between competitive and non-competitive inhibition.

The degree of enzyme inhibition caused by a non-competitive inhibitor is dependent solely on the amount of enzyme combined with the drug, and therefore the only equilibrium to be considered is that concerned with formation of enzyme-inhibitor complex.

$$E + I \rightleftharpoons EI$$

Let I = concentration of inhibitor

e = initial concentration of enzyme

q = concentration of enzyme-inhibitor complex

K_I = dissociation constant of enzyme inhibitor complex

i = fractional inhibition

$$= \frac{q}{e}$$

a = fractional activity, where $1 - a = i$.

By the law of mass action, assuming only a negligible fraction of total inhibitor present is combined with enzyme,

$$I(e-q) = K_I.q$$

$$\therefore \quad \frac{I(e-q)}{q} = K_I$$

substituting $q = ei$

$$\frac{I(1-i)}{i} = K_I$$

substituting for $i = 1-a$

$$I\frac{a}{1-a} = K_I \quad . \quad . \quad . \quad . \quad . \quad (7)$$

This treatment has been applied by Hunter and Downs (1945). Equation 7 provides a method for determining K_I, and also shows that, with given enzyme and inhibitor concentrations, the residual enzyme activity (*i.e.* degree of inhibition) is constant and independent of substrate concentration, and only dependent on the dissociation constant of enzyme-inhibitor complex.

The kinetics of competitive inhibition were worked out by Michaelis and Menten (1913). In this case, two reversible reactions are to be considered, formation of enzyme-substrate complex, and of enzyme-inhibitor complex.

$$E + S \rightleftharpoons ES \qquad E + I \rightleftharpoons EI$$

Let S = concentration of substrate

I = ,, ,, inhibitor

p = ,, ,, enzyme-substrate complex

q = ,, ,, enzyme-inhibitor complex

K_s = Dissociation constant of enzyme-substrate complex

K_I = Dissociation constant of enzyme-inhibitor complex.

Then, by the law of mass action,

$$S(e-p-q) = K_s\, p$$
$$I(e-p-q) = K_I\, q$$

eliminating q we get

$$K_I = \frac{I\,K_s}{S\left(\frac{e}{p}-1\right)-K_s} \quad . \quad . \quad . \quad (8)$$

Let v_0 = velocity of enzymic reaction without inhibitor
v = velocity of enzymic reaction in presence of inhibitor
p_0 = concentration of enzyme-substrate complex in absence of inhibitor.

In each case the velocity of reaction is proportional to the concentration of enzyme-substrate complex.

i.e. $$\frac{v_0}{v} = \frac{p_0}{p} \quad \therefore\ p_0 = \frac{v_0}{v}\,p.$$

Now, a value for p_0 has already been derived as $\frac{eS}{K_s+S}$ (p in equation (1) p. 150).

$\therefore$ $$\frac{v_0}{v}\,p = \frac{eS}{K_s+S}$$

$$\frac{e}{p} = \frac{v_0}{v} \cdot \frac{K_s+S}{S}$$

Substituting for $\frac{e}{p}$ in equation (8)

$$K_I = \frac{I \cdot K_s}{S\left(\frac{v_0}{v} \cdot \frac{K_s+S}{S}-1\right)-K_s}$$

$$= \frac{I \cdot K_s}{\frac{v_0}{v}(K_s+S)-S-K_s}$$

$$K_I = \frac{I \cdot K_s}{(K_s+S)\left(\frac{v_0}{v}-1\right)} \quad . \quad . \quad . \quad . \quad (9)$$

Hunter and Downs (1945) substitute the term fractional activity (a) for $\frac{v}{v_0}$ in equation (9), which becomes

$$K_I = \frac{I \cdot K_s}{(K_s+S)\left(\frac{1}{a}-1\right)}$$

or

$$I \cdot \frac{a}{1-a} = \frac{K_I}{K_s}(K_s+S)$$

$$I \cdot \frac{a}{1-a} = K_I + \frac{K_I}{K_s} S \quad . \quad . \quad . \quad (10)$$

Substituting C for $K_I + \frac{K_I}{K_s} S$, we get

$$I \frac{a}{1-a} = C \quad . \quad . \quad . \quad . \quad (11)$$

This equation is comparable with equation (7) obtained for non-competitive inhibition, except that, in this case, C is not a constant but is proportional to substrate concentration. The extent of inhibition is also proportional to the relative values of dissociation constants K_I and K_s. In other words, equation (10) shows us that the characteristic properties of competitive inhibition are variation of inhibition with substrate concentration and dependence on the relative affinities of the enzyme for substrate and inhibitor. If $I \cdot \frac{a}{1-a}$ is plotted against S, the graph will be a straight line sloping upward from the axis of S (Fig. 20). Its intercept on the $I \cdot \frac{a}{1-a}$ axis is K_I and its slope is $\frac{K_I}{K_s}$, from which values both K_I and K_s may be calculated. The term $I \cdot \frac{a}{1-a}$ represents a useful yardstick for relating the effects of different inhibitors. It is numerically equal to the concentration of inhibitor by which activity of a given enzyme is reduced to one-half of its original value.

Adequate study of enzyme inhibitors cannot be carried on without investigation of the kinetics of inhibition. This is now being realised to an increasing extent by workers in the field, who, however, often appear to fail to realise the value of the use of pure enzymes in their experiments. Much painstaking work on impure enzymes has subsequently been proved to be valueless when kinetic data were provided for the pure enzymes.

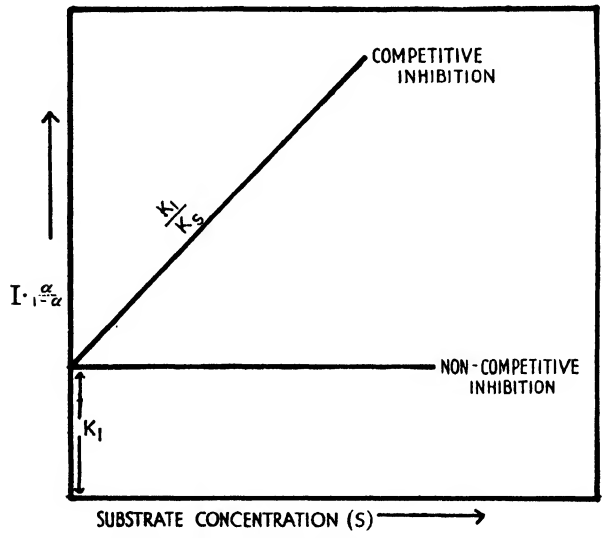


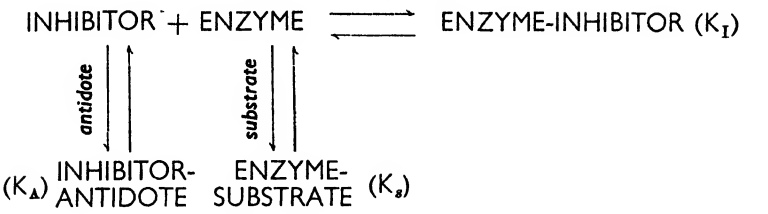
FIG. 20.—Characteristic graphs of competitive and non-competitive inhibition. I = inhibitor concentration, α = fractional activity, where $1-\alpha$ = degree of inhibition. (Enables calculation to be made for values of K_I, dissociation constant of enzyme-inhibitor complex, and K_s, dissociation constant of enzyme-substrate complex.)

Kinetic treatment has been applied to the sulphonamide inhibition of growth of bacterial cells and its competitive reversal by *p*-aminobenzoic acid (Klotz and Gutmann, 1945). This enabled the dissociation constants (K_I) of the drug-enzyme complexes to be estimated.

Reversal of inhibition

When inhibition of an enzyme is reversible, the enzyme-

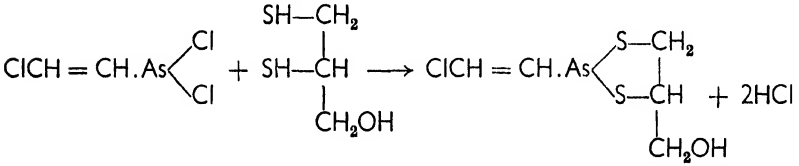
inhibitor complex is in equilibrium with the dissociated constituents according to the equation



At equilibrium, the extent of dissociation will depend on the dissociation constant (K_I) of the enzyme-inhibitor complex and will increase with increasing values of K_I. Equilibrium may be altered either by removal of enzyme by combination with additional substrate, or by removal of inhibitor through chemical combination with an antidote. The enzyme-inhibitor complex will then dissociate further in an attempt to restore the equilibrium, and some degree of reversal of inhibition will be effected. In the absence of antidote, the effectiveness of an inhibitor depends, as we have seen (equations 7 and 10), on the values of K_s and K_I.

Chemical combination of inhibitor with an antidote may provide an effective method of reversing enzyme inhibition. If the inhibitor-antidote complex is very insoluble, the reversal of inhibition is complete, since all the inhibitor is removed from solution. Such is the case in the reversal by hydrogen sulphide of the inhibition by heavy metals of " SH enzymes." Complete reversal could also be attained if the dissociation constant (K_A) of the inhibitor-antidote complex were very low compared with that of enzyme-inhibitor complex (K_I). Recent investigation on war gases has clearly demonstrated the dependence of toxic action upon the dissociability of poison-enzyme and poison-antidote complexes. The vesicant lewisite, and lachrymators such as chloracetophenone and bromobenzyl cyanide, were found to be powerful inhibitors of all " SH enzymes," *i.e.* low values of K_I (Dixon and Needham, 1946 ; Peters, Stocken and Thompson, 1945). Among a series of related lachrymators, the greatest degree of enzyme inhibition was obtained with those compounds which formed

most stable addition products with thiols. Inhibition by lewisite of pyruvate oxidase or hexokinase could not be reversed by monothiols, such as cysteine or glutathione, which reacted with the poison to give compounds dissociating in dilute solution (K_A high). The dithiol 2:3-dimercaptopropanol (British anti-lewisite or B.A.L.) reacted with lewisite to form a highly stable non-dissociating ring compound (K_A low) and was completely effective in reversing the action of lewisite on pyruvate oxidase or other SH-enzymes (Stocken and Thompson, 1946; Whittaker, 1947; Barron *et al.*, 1947). B.A.L. was found to be effective not only in reversing the *in-vitro* inhibition of " SH enzymes " by lewisite, but also in



reversing the toxic and vesicant action of lewisite *in vivo* for at least an hour after contamination of the skin.

If K_A is equal to or slightly greater than K_I, fairly effective reversal of inhibition may be attained by the use of a large excess of antidote over inhibitor. The reversal by cysteine or glutathione of " SH inhibition " by arsenicals or mercurial compounds is an example of this type of reversal; reactivation of succinoxidase by glutathione after poisoning by *p*-carboxyphenylarsenoxide required a glutathione concentration fifty times greater than that of the inhibitor (Barron and Singer, 1945*a*).

In competitive inhibition, where the inhibitor is antagonised by the substrate, we know that the relative values of K_I and the enzyme-substrate dissociation constant (Michaelis constant, K_s) play an important part in determining the efficiency of an inhibitor. An ideal competitive inhibitor would be one whose affinity for the enzyme is considerably greater than that of the substrate, otherwise the ratio of inhibitor/substrate concentrations would have to be high. In the case of inhibition

of succinic dehydrogenase by malonic acid, we have a high affinity between substrate analogue and enzyme, the inhibitor/substrate ratio for 50 per cent. inhibition being as low as 1/50 (Potter and DuBois, 1943). This high affinity represents an exception, rather than the rule, and in most cases of competitive inhibition so far examined, K_s, is found to be lower than K_I. This might be expected in view of the high specificity shown by an enzyme for its natural substrate; it implies that reversal of drug inhibition can be effected, in certain cases, by increase in substrate concentration, an undesirable character in a chemotherapeutic drug. Kinetic data provided for *d*-amino acid oxidase (unfortunately on impure enzyme preparations), show that quinine inhibits the enzyme by competing with the coenzyme flavin adenine dinucleotide (Hellerman, Lindsay and Bovarnick, 1946). The ratio of the two dissociation constants K_I/K_s was about 1000, K_I being about 4×10^{-4} and K_s 4×10^{-7}. With a quinine concentration of 10^{-3}M, 61 per cent. inhibition was obtained with a coenzyme concentration of $0{\cdot}4\times10^{-7}$M; when the coenzyme concentration was raised to $27{\cdot}5\times10^{-7}$M the degree of inhibition was reduced to 10 per cent. Here we see that because of the enormous difference between the dissociabilities of the complexes of enzyme with inhibitor and coenzyme, a very small amount of additional coenzyme in the reaction system largely eliminated the inhibition caused by a relatively high concentration of quinine.

Certain combinations between active centre and inhibitor may not be reversible. In this case, substrate or coenzyme can only protect against inhibition when added prior to the inhibitor, but the degree of inhibition will still be proportional to the concentration ratio. This condition has been found by Haas (1944) to occur in the inhibition by atebrin of cytochrome reductase and glucose - 6 - phosphate dehydrogenase. The reversibility of atebrin-enzyme combination appears to vary with different enzymes, since Hellerman reports that inhibition of pancreatic lipase by atebrin shows strictly reversible competition with substrate. With *d*-amino acid oxidase he suggests that the effect of atebrin might be explained by a

slow irreversible combination superimposed on a strictly reversible reaction (Hellerman, Lindsay and Bovarnick, 1946). In the inhibition of SH-enzymes by heavy metals, increase in the concentration of metal may decrease the degree of reversal attained by addition of thiol compounds (Sumner and Myrbäck, 1930; Barron and Kalnitsky, 1947). This may be due to irreversible denaturation of the enzyme by the heavy metal.

Effect of inhibitors on living cells

The foregoing sections of this chapter have dealt in some detail with the inhibition of isolated enzymes. This is necessary, because any adequate consideration of mode of drug action in the living cell must be related to these facts. If, for example, a drug has a grouping which can combine with sulphydryl groups and its effect is reversed by an excess of cysteine, it is reasonable to assume that it is inhibiting some enzyme dependent for activity on free sulphydryl groups. The action of another drug might be reversed by a known metabolite in such a way that the ratio of drug to metabolite is constant for any given degree of inhibition; in this case the drug may be acting by competing with the metabolite for the enzyme whose substrate is that particular metabolite. These suppositions would be strengthened by investigating the effects of the drug on isolated enzymes of the types suggested by *in-vivo* experiments. However, a positive result from such experiments should not be taken as proof that the action on the particular enzyme is the *only* action of the drug *in vivo*, unless the importance of that enzyme in cell economy is known.

An indication of the importance of an enzyme to the cell may be obtained from a knowledge of the cellular concentration of enzyme and the rate at which it reacts with its particular substrate. A measure of this rate is given by the constant known as *Turnover Number*, which may be defined as the number of molecules of substrate (or coenzyme) which will undergo reaction with one molecule of enzyme in one minute when *p*H and substrate concentration are such that the

enzyme is working at its maximum velocity for the temperature under consideration. In the case of a respiratory enzyme, a knowledge of enzymic concentration and Turnover Number enables one to calculate the maximum fraction of total respiration which could pass through the pathway catalysed by that enzyme. The percentage inhibition of total cell respiration due to the action of the inhibitor on that pathway could then be calculated, and compared with the experimental value.

Unfortunately, enzymology has not progressed far enough for us to find in the literature all the data to enable such calculations to be made for most enzymes. The determination of the absolute amount of an enzyme in cells is a measurement which can be made with certainty only in a few cases, because of the difficulty of quantitative extraction from the cell. The difficulty in measuring Turnover Number is not so great, but in spite of this, insufficient data have been collected up to the present for calculation of Turnover Numbers of different enzymes when acting on *substrates actually present in the living cell.* McIlwain (1946) has estimated from available data the Turnover Numbers of various enzymes and also the amounts contained in a single bacterial cell. He concludes that certain enzymes, possibly associated with genes or cellular synthesis, must exist in concentrations of only one or a few molecules per cell; others exist in very much higher concentrations (see also Herbert and Pinsent, 1947).

In the case of cytochrome *c*, we are fortunate in having a protein whose activity and concentration can be measured in the intact cell by means of its absorption spectrum. This was first done for yeast by Haas (1934) by measuring with a photoelectric spectrophotometer the light absorption at 550 mμ of a washed yeast suspension. This wave-length represents the position of the strongest visible absorption band of reduced cytochrome *c*, and its intensity gives a measure of the amount of reduced cytochrome *c* present at any given time. Comparison of the absorption at 550 mμ of the yeast in a nitrogen atmosphere with that of a standard solution of reduced cytochrome *c* gave the concentration of cytochrome

as $1{\cdot}43\times10^{-5}$ m.M per ml. of yeast suspension. The rate of reduction of oxidised cytochrome *c* was determined by measuring, in the presence of cyanide, the rate of change of absorption of a fully-oxygenated suspension. Cyanide acts by inhibiting cytochrome oxidase, so the overall visible change was due only to conversion of oxidised cytochrome to the reduced form.

The velocity constant K for the reduction was calculated from the expression

$$K = \frac{1}{t} ln \frac{Co}{C}$$

where t = time in minutes.

Co = concentration of oxidised cytochrome at time $t = 0$.

C = concentration of oxidised cytochrome at time t.

K was found to be 4. This means that the volume of oxygen bound by cytochrome oxidase when acting through cytochrome *c* would be

$4 \times \frac{22{,}400}{4} \times 1{\cdot}43\times10^{-5} = 0{\cdot}32$ mm.^{3} O_2 per ml. of cell suspension per minute, since 1 mol. of cytochrome is equivalent to $\frac{1}{4}$ of mol. of oxygen (p. 52). Manometric determination of oxygen consumption of the same yeast suspension gave a value of 0·34 mm.^{3} O_2 per ml. of cell suspension per minute. This shows that, under the conditions of the experiment, practically all the respiration of yeast passed through the cytochrome oxidase-cytochrome *c* system, and that the inhibitory effect of cyanide could be fully accounted for by its inhibition of cytochrome oxidase.

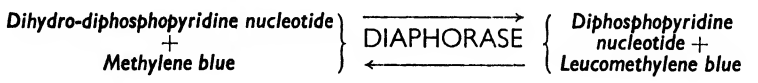
Having evidence that cytochrome is capable of transporting all the hydrogen which combines with oxygen in yeast respiration, Warburg (1934) calculated the Turnover Number of cytochrome from the cytochrome content and the oxygen uptake of the cells. The value for yeast worked out at 4000. Keilin (1940) calculated the Turnover Number of cytochrome *c* in yeast by a different method and obtained a value of 3850. It is interesting to compare these values obtained for intact cells, with the figure of 1420 given by Keilin for a cell-free

colloidal system containing the complete cytochrome system and succinic dehydrogenase. The much lower figure for the cell-free system indicates, as Keilin points out, "that the great efficiency of catalytic systems within living cells is due to a high structural organisation responsible for the proper spatial distribution and the most favourable molecular orientation of all components of the system." Methods have not yet been developed by which Turnover Numbers of most other enzymes can be measured in the intact cell, and it is probable that any value found *in vitro* represents a figure considerably below the *in-vivo* figure.

Negelein and Wulff (1937) provide data showing that *in vitro* one molecule of alcohol dehydrogenase can reduce at least 1800 molecules of pyridine nucleotide per minute. As far as Turnover Number is concerned, concentration of pyridine nucleotide in the living cell is highly misleading, since nucleotide coenzymes usually occur in large excess over the corresponding dehydrogenase proteins (Schlenk, 1942). Working with nicotinamide-deficient *Proteus vulgaris* produced by growth in low concentrations of nicotinamide, Morel (1941) estimated the concentration of nicotinamide in the cells when they first showed symptoms of nicotinamide deficiency. At this stage it is probable that all the coenzyme present was used for hydrogen transport. Measurement of the reducing power of such cells for methylene blue in the presence of glucose, gave a Turnover Number for nicotinamide dehydrogenases oxidising glucose *in vivo* as 600. As pointed out by Morel, it is unlikely that all hydrogen transport is mediated by the nicotinamide coenzymes, so that the figure given is a maximum for the conditions used. The value is considerably lower than that given by Negelein and Wulff for alcohol dehydrogenase, but this is to be expected, since the value arrived at by Morel represents an overall figure for depleted cells and for a whole group of dehydrogenases, some of which may have much lower Turnover Numbers. *In vivo*, moreover, there is no guarantee that at each stage in a series of linked dehydrogenations, each enzyme will be saturated with the appropriate substrate.

The Turnover Number of a flavoprotein enzyme in living *Lactobacillus delbruckii* was calculated by Warburg (1934) from unpublished figures obtained by Haas. This organism normally grows under anaerobic conditions; it has no cytochrome or other hæme pigments and is not affected by cyanide; in the presence of oxygen, however, it takes up oxygen and produces hydrogen peroxide. Haas observed a visible absorption band at 460 mμ under aerobic, but not under anaerobic, conditions, and concluded it was due to a flavoprotein. The rate of oxidation of the flavoprotein was calculated by observing anaerobically appearance of the band after addition of methylene blue. The corresponding oxygen uptake due to this enzyme was calculated to be 1·2 mm.^{3} O_2 per ml. suspension per minute, while the observed oxygen uptake was 1·0. Warburg concluded that all respiration of this organism, observed when it is kept under aerobic conditions, can pass through a flavoprotein. The Turnover Number for the enzyme was calculated to be 30. This is a very low value for a respiratory enzyme, but since the organism does not normally exist aerobically we may infer that the flavoprotein is not playing its normal role under these circumstances. A valuable extension of this work would be a determination of the Turnover Number of the flavoprotein under anaerobic conditions with no artificial hydrogen acceptor.

The flavoprotein diaphorase has been found to have *in vitro* a Turnover Number of 8000 when it is transferring hydrogen in the following system (Straub, Corran and Green, 1939; Straub, 1942):—



Although this value was not determined with the natural hydrogen acceptor of diaphorase, since it is unknown, there is evidence that this reaction rate is of the same order as in the intact cell. Unfortunately there are no figures available for the diaphorase content of cells, so that the proportion of respiration passing through diaphorase cannot be estimated.

The position is different with regard to cytochrome reductase, the other known flavoprotein concerned in hydrogen transport from pyridine nucleotide dehydrogenases to cytochromes. Here, in the case of yeast at least, a figure for its concentration has been given (Haas, Horecker and Hogness, 1940), and its Turnover Number has been estimated as 300 to 500 (Green, 1941). A Turnover Number of about 400 would seem to be a reasonable figure to use in an attempt to estimate the part played by this enzyme in respiration of bakers' yeast at 28°.

The rate of respiration of yeast expressed as respiratory coefficient, Q_{O_2}, is 60-80 at 28° (Q_{O_2} = mm.^{3} of oxygen used per mg. dry weight per hour). This means that 1 kg. dry weight of yeast takes up 0·045 gm. mol. of oxygen per minute. According to our calculation, 1 mol. of cytochrome reductase can in one minute transfer 400 mols. of hydrogen, *i.e.* can reduce 200 mols. of oxygen. The molecular weight of the enzyme is 75,000, and 1 kg. dry yeast contains 0·6 g. of enzyme, *i.e.* $\frac{0{\cdot}6}{75{,}000}$ gm. mol. Therefore, number of gm. mols. of oxygen taken up per minute by 1 kg. of yeast through cytochrome reductase $= \frac{0{\cdot}6 \times 200}{75{,}000} = {\cdot}0016$. This shows that only $\frac{{\cdot}0016}{{\cdot}045}$, *i.e.* 3·5 per cent., of the total oxygen uptake of yeast can be carried on by means of the glucose-6-phosphate dehydrogenase-cytochrome reductase system if this is the only system in which cytochrome reductase plays a part. Our knowledge of cellular metabolism is still so slight that we are unable to say whether cytochrome reductase acts as hydrogen acceptor to any enzyme other than glucose-6-phosphate dehydrogenase. Neither are we able to estimate the importance in normal aerobic respiration of this practically unknown route of carbohydrate oxidation, which is not the normal anaerobic glycolytic pathway about which more is known (pp. 68 and 72).

It is, however, instructive to examine the type of information we can obtain from these calculations in the

unlikely circumstances that all our assumptions are correct. We may say that any inhibitor acting exclusively on the glucose - 6 - phosphate dehydrogenase cytochrome reductase system would reduce respiration of yeast by 3·5 per cent. In the present state of knowledge, it is impossible to say whether an inhibitor acts only on one enzyme, but we may examine inhibitors known to act on the cytochrome reductase system

TABLE 11

Effect of inhibitors on isolated respiratory enzymes and on respiration of intact cells

System Studied	Per cent. Inhibition by		
	10^{-3}M 2 : 4-dinitro-*o*-cyclohexyl phenol	5×10^{-4}M Atebrin	5×10^{-4}M Quinine
Glucose-6-phosphate / TPN } Dehydrogenase	90	78	0
TPN / Cytochrome *c* } Cytochrome reductase	70	73	14
Cytochrome *c* / Oxygen } Cytochrome oxidase	0	40	17
Respiration of bakers' yeast .	93	...	0 (17 per cent. inhibition by $1{\cdot}3 \times 10^{-4}$M) *
Respiration of malaria parasite .	...	80 †	26 †

Figures from Haas (1944), except for * Rona and Grassheim (1923), † Fulton and Christophers (1938).

and compare their effects on the isolated enzyme and on cellular respiration. Haas (1942) found that 2 : 4-dinitro-*o*-cyclohexylphenol has an inhibitory effect on cytochrome reductase in concentrations of ·001M ; the same concentration also inhibited glucose-6-phosphate dehydrogenase but not cytochrome oxidase (see Table 11). We have to assume that cytochrome reductase is the only hydrogen carrier acting between glucose-6-phosphate dehydrogenase and cytochrome,

and therefore inhibition of either enzyme will have the same overall effect on respiration. We have calculated that this effect should be a 3·5 per cent. reduction of respiration. Haas found that ·001M 2 : 4-dinitro-*o*-cyclohexylphenol reduced respiration of yeast by 93 per cent., which suggests to us that the phenol also inhibits some other respiratory enzymes.

Experiments were also carried out by Haas (1944) on the effect of the antimalarial drugs atebrin and quinine on the enzymes involved in the oxidation of glucose-6-phosphate ; these are compared in Table 11 with results of Rona and Grassheim (1923) and Fulton and Christophers (1938) on respiration of yeast and the malaria parasite. The concentrations used were of the same order as those occurring in the blood stream after administration of therapeutic doses. The results indicate that atebrin has a far greater inhibitory effect both on total cell respiration, and on the respiratory enzymes investigated, than has quinine. Haas interprets his results as showing that, among the known components of the respiratory system, only cytochrome reductase and glucose-6-phosphate dehydrogenase need be considered as possible points of interference by atebrin in the malaria parasite. This can only apply if the whole respiration goes through the cytochrome reductase pathway. Little is known of the respiratory mechanism of the malaria parasite, which seems, however, to conform to the general scheme of carbohydrate metabolism (Bovarnick, Lindsay and Hellerman, 1946 *a* and *b* ; Evans, 1946 ; Speck and Evans, 1945*a*). The importance of the cytochrome reductase system is quite unknown, and Haas was unable to estimate the concentration of cytochrome reductase in parasites. Therefore Haas' assumption is unjustified, particularly as he himself shows that cytochrome oxidase, a very important respiratory enzyme, is inhibited by 40 per cent., a result confirmed by Hellerman, Bovarnick and Porter (1946).

Work by Hellerman, Lindsay and Bovarnick (1946) has shown that therapeutic concentrations of atebrin and quinine inhibit many other enzyme systems and may be regarded as fairly general enzyme poisons. The site of their attack on the living malaria parasite cell is therefore even more obscure,

particularly as many non-antimalarial quinolines were also shown to be effective enzyme inhibitors. However, with parasites initially depleted of glucose, the respiration resulting from addition of glucose is inhibited 75 to 90 per cent. by ·0001M atebrin; this inhibition can be reversed by adenylic acid or adenosine triphosphate in a manner suggesting competition. Quinine and plasmoquine had similar effects, and in all cases the inhibitory concentrations had no effect on cells not initially deprived of glucose (Bovarnick, Lindsay and Hellerman, 1946*b*). This suggestion that utilisation of adenosine triphosphate is inhibited by antimalarial drugs agrees with the finding of Speck and Evans (1945*b*) that atebrin inhibited hexokinase more strongly than any of the other known enzymes of carbohydrate metabolism. The possibility cannot be excluded that in all these cases combination occurred between basic drug and the acidic adenosine triphosphate, so that the drug was having no direct effect on the enzyme.

It must now be evident that there is great need for quantitative data on the amounts of various enzymes present in different micro-organisms and tissues, and on the Turnover Numbers of the reactions catalysed by these enzymes *in vivo*. Until such data are available, attempts to prove on a quantitative basis that a particular drug owes its activity to inhibition of a particular enzyme are of doubtful validity. Such attempts do, however, serve a useful purpose in indicating in which direction more knowledge must be sought, and in suggesting other methods of quantitative attack on the same problem based on the effect of drug antagonists (see Chapter V).

Reduction of respiration of an organism probably implies that an inhibitor is acting on one or more of the enzymes concerned with carbohydrate breakdown. Some indication of the site of action in the intact cell may sometimes be obtained by demonstration of accumulation of an intermediate product normally metabolised by the cell. Such a case is the strong inhibition of carbohydrate breakdown by fluoride, in which there is an accumulation of 2-phosphoglyceric acid (see p. 161); here it is possible to say that the major effect of

fluoride is on enolase which acts on phosphoglyceric acid. Inhibition of oxidation or fermentation of one carbohydrate, for example glucose, but not of some of its normal breakdown products such as pyruvate or lactate, may suggest that the site of inhibition is on the pathway between glucose and pyruvate. A change in anaerobic breakdown product may also indicate blockage of one method of breakdown; for example, if a fermenting organism which normally produces mostly acetic acid from added pyruvic acid produces lactic acid under the influence of an inhibitor, one may surmise that the path from pyruvic to acetic acid is blocked, but that the cell is able to utilise an alternative pathway for energy production (see p. 267 for specific examples).

This use of alternative metabolic pathways under the influence of enzyme inhibitors may play an important part in the overall effect of inhibitors on cells. For example, two organisms may react differently to the same inhibitor because one may have an alternative pathway available and so can bypass the inhibited step, while the other will suffer some adverse effect through inability to utilise another pathway. The forcing of a reaction through an alternative pathway can also be imagined to be harmful to an organism, since the alternative route may not be a complete substitute for the normal reaction.

Work by Hotchkiss (1944) on the antibiotic gramicidin, suggests that it may act on micro-organisms by preventing normal energy exchange through stimulating an abnormal non-phosphorylative type of carbohydrate breakdown. Gramicidin, like many other chemotherapeutic agents, has a bacteriostatic rather than a bactericidal action at the concentration used therapeutically. This could result from the inability of the poisoned cell to utilise energy to force synthetic reactions required for growth and cell division. Hotchkiss found that gramicidin, dinitrophenol and azide all increased the rate of respiration of *Staph. aureus* but inhibited the uptake of inorganic phosphate normally associated with respiration. Growth of yeast is not so readily inhibited by gramicidin as is that of *Staph. aureus*; yeast is, however, susceptible to

inhibition by dinitrophenol, and here too, uptake of inorganic phosphate and synthesis of polysaccharide were inhibited by this drug. Dinitrophenol and azide probably prevent utilisation of metabolic energy for synthetic processes by inhibiting certain energy-rich phosphate transfers without inhibiting carbohydrate breakdown (Clifton and Logan, 1939; Winzler, Burk and du Vigneaud, 1944; Spiegelman, Kamen and Dunn, 1946). The fate of "labelled" phosphorus in normal yeast was investigated by Spiegelman and Kamen (1946), who showed that during protein synthesis there was a fall in nucleoprotein phosphorus. No such fall occurred when fermentation was allowed to proceed in the absence of a source of nitrogen, when there was no growth or protein synthesis. Under conditions otherwise suitable for protein synthesis, dinitrophenol and azide prevented the fall in nucleoprotein phosphorus (review, McElroy, 1947).

The importance of phosphorylation as a mechanism for energy storage and transfer has already been stressed. So far as we know, energy-rich phosphate bonds are the main energy store available for the forcing of desirable endergonic reactions, and any substance capable of preventing the formation or utilisation of phosphate bonds may prevent cell synthesis. Spiegelman and Kamen suggest that nucleoprotein phosphorus may be intimately concerned with cell synthesis, so that interference in its metabolism may prevent growth. Penicillin also seems to act by preventing cell synthesis rather than by interference with the oxidative energy-yielding mechanisms, and it may be significant that nucleic acid metabolism is susceptible to inhibition by this drug (Krampitz and Werkman, 1947). The passage of glutamic acid across the cell wall of *Strep. fœcalis* is also dependent on a supply of energy and is inhibited by penicillin. Gale and Taylor (1946*b*, 1947*b*) suggest that this may be the primary site of penicillin inhibition (see p. 272 for full discussion).

Since many other chemotherapeutic drugs prevent the growth of susceptible organisms rather than kill them outright, it is evident that they could also act by preventing synthetic rather than degradative reactions. This could be

effected either by inhibition of energy-producing reactions or by inhibition of synthetic processes themselves. The point is illustrated by the following example : growth of *Escherichia coli* in a synthetic ammonium chloride medium was accompanied by uptake of ammonium ion from the medium. This fixation of nitrogen required an oxygen consumption about 55 per cent. above that of resting cells. The resting respiration was scarcely affected by concentrations of sulphathiazole which completely inhibited growth, but the additional respiration associated with nitrogen fixation and cell synthesis was as susceptible to inhibition by sulphathiazole as was growth itself (Armstrong and Fisher, 1947 ; Fisher and Armstrong, 1947). These experiments do not indicate that sulphathiazole inhibits growth by inhibition of a sulphonamide-sensitive respiratory system associated with growth ; they simply indicate a trap for the unwary—confusion of cause and effect. There is little doubt, as we shall show, that the primary site of sulphonamide inhibition is in the conversion of *p*-aminobenzoic acid to pteroylglutamic acid. The degree of inhibition of sulphonamide-sensitive respiration is probably a measure of the lessened need of the cell for exergonic oxidative reactions when its endergonic synthetic reactions are inhibited. As we pointed out previously, very little is known about these synthetic reactions, so our lack of direct knowledge on the mode of action of chemotherapeutic drugs is not surprising.

As far as we know, the main energy-producing pathways employed by most animals and heterotrophic micro-organisms are similar. This may be attributed to the fact that both forms of life derive most of their energy from the carbohydrates, and that differentiation during evolutionary development has been more concerned with change in synthetic pattern than with change in energy source for building that pattern. Chemotherapy is concerned with destruction of invading micro-organisms in the tissues of the host ; this means that a successful chemotherapeutic drug attacks the micro-organism but not the host. This implies that the enzymes in the micro-organism which are inhibited by the drug may have different essential groups from those in the

host; alternatively, the susceptible enzymes may be either absent from the host tissues to which the drug has access, or of relatively less importance to the host. A successful chemotherapeutic drug, *i.e.* one that is completely non-toxic to the host, is therefore unlikely to inhibit strongly any part of carbohydrate breakdown common to both host and infective agent, unless an effective alternative path is available to the host. A completely rational approach to chemotherapy could only be achieved after acquisition of full knowledge of all the enzyme systems of animals and micro-organisms. This is a goal for the future. At present we can only examine the effects on organisms and isolated enzymes of our known chemotherapeutic drugs, and from a knowledge of the principles of enzyme inhibition described in this chapter, make attempts at guessing their mode of action.

CHAPTER V

DRUG ANTAGONISM

SINCE enzyme inhibition is often a reversible process, it is not surprising that the growth-inhibitory action of drugs on living cells can frequently be reversed by addition to the drug-cell system of a third component which can bring about removal of the drug from its site of action. The simplest of all examples of drug antagonism, and one which is of such daily occurrence that its nature is generally ignored, is the antagonism between hydroxyl and hydrogen ions. Growth of a micro-organism in the richest media is prevented by the addition of excess alkali (drug), but the antibiotic action of alkali may be antagonised by addition of a suitable quantity of acid (antagonist), and cell growth is resumed, provided, of course, that the micro-organism has not been left for too long in the alkaline medium so that irreversible denaturation of cellular protein has occurred.

Antagonism may be direct, in that the antagonist combines chemically with the drug and forms a physiologically inactive drug-antagonist complex, or it may be indirect, in that no chemical interaction between drug and antagonist is possible but each is capable of displacing the other from its biological point of action. Several examples of such indirect antagonism in isolated enzyme systems have been described in the previous chapter as competitive inhibitions. The conditions under which competitive inhibition can be expected have been described and criteria for the competitive nature of an inhibition have been laid down (Chapter IV).

It will be remembered that competitive inhibition has been found to occur when an enzyme system is treated with a compound which is structurally similar to a natural substrate and which can therefore " fit " the active centre of the enzymic protein. All essential metabolites, whether they function as coenzymes, prosthetic groups, activators or building blocks

are in the last analysis substrates, in the sense that some protein structure in the cell is specifically designed to " fit " their particular electronic configuration. Any substance, made available to the living cell, which penetrates the cell wall and is closely similar in electronic configuration to an essential metabolite (organic or inorganic), will be liable to be caught up in the metabolic wheel at the point specifically designed to accommodate the related metabolite. If the structure of the metabolite analogue is such that it can undergo chemical changes similar to those undergone by the natural metabolite, but at a slower rate, then the living cell may utilise the analogue as an unsatisfactory substitute and grow at a reduced rate limited by the Turnover Number of the modified enzyme system. If the metabolite analogue " fits " the enzyme but cannot undergo conversion to a functional form, then it may act as an enzyme inhibitor and hence as a growth inhibitor. The nature of its mode of action will be indicated by the capacity of the natural metabolite to act as an antagonist in a competitive manner.

Some confusion of thought has arisen from the above argument. Although a metabolite analogue may act as a growth inhibitor by displacing the natural metabolite, it does not follow that every substance capable of reversing the inhibitory action of a drug is an essential metabolite. Indeed, the capacity to antagonise the growth-inhibitory action of a drug does not in any way constitute evidence for the metabolic rolé of the antagonist, even when the drug-antagonist relationship follows all the laws of competitive inhibition. Evidence for the metabolic function of a drug antagonist must come from studies on metabolism, rather than from studies on drug antagonism.

The drug antagonisms of classical pharmacology were well recognised long before the subject became of immediate interest in the biochemical interpretation of chemotherapy ; it is not possible to give a detailed account of the work here, and the monograph by A. J. Clark (1937) should be consulted as a guide to this field. " Therapeutic interference " was the term used by Browning to describe the reduction in trypano-

cidal action which could be caused by injection of a second drug after feeding of a trypanocidal drug (see p. 230). Not until the sulphonamides assumed a leading place in chemotherapy and their mode of action was subjected to intensive study, did therapeutic interference and drug antagonism begin to shed light upon the enzymic interpretation of drug action.

Sulphonamides and p-*aminobenzoic acid*

The reversal of the bacteriostatic effect of sulphonamides by peptone was noted by Lockwood (1938). In the following

TABLE 12

Concentration of antagonist required to reverse growth inhibition of Strep. hæmolyticus *caused by sulphanilamide*

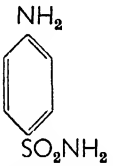
Concentration of sulphanilamide ($M \times 10^{-3}$) (*a*)	Ml. of extracted antagonist ($\times 10^{-1}$) (*b*)	Ratio b/a	Concentration of *p*-aminobenzoic acid ($M \times 10^{-7}$) (*c*)	Ratio $c/a \times 10^{-4}$
0·303	0·016	5·3	0·58	1·92
1·515	0·08	5·3	2·91	1·92
7·575	0·4	5·3	14·54	1·92

(Woods, 1940.)

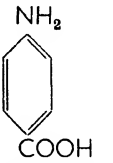
year, Stamp (1939), in seeking for other sulphonamide antagonists, found that a concentrated extract from hæmolytic streptococci was about a hundred times more active than peptone. Stamp suggested that his substance might be an essential metabolite for bacteria and an essential part of an enzyme system which was inhibited by sulphanilamide. Green (1940) extracted from culture media filtrates of *Brucella abortus*, and from the organism, a factor which reversed the bacteriostatic action of sulphanilamide. He suggested, also, that this factor acted by stimulating some enzyme system in the bacterial cell which was inhibited by sulphanilamide. Soon afterwards, Woods (1940) showed that a sulphanilamide antagonist, prepared in much the same way as Stamp's

concentrate, contained an amino derivative of an aromatic carboxylic acid. This antagonist was also shown to be obtainable from various plant and animal tissues. It antagonised sulphanilamide in a competitive manner, since the ratio of the amount of antagonist to sulphonamide was constant for various sulphonamide concentrations (see Table 12).

Competition between substances of closely related chemical structure in enzymic reactions was already well recognised, and suggested to Woods that the active fraction of his extract was closely related in chemical structure to sulphanilamide. Accordingly, he tested a series of compounds related to sulphanilamide for antagonistic action, and found that *p*-aminobenzoic acid at a concentration of 1·2 to $5{\cdot}8 \times 10^{-8}$M was sufficient to reverse the inhibition caused by $3{\cdot}03 \times 10^{-4}$M sulphanilamide. As with yeast extracts, there was a constant ratio between the concentration of sulphanilamide and the concentration of *p*-aminobenzoic acid required to reverse the inhibition (Table 12).



Sulphanilamide



p-Aminobenzoic acid (P.A.B.A.)

Of sixteen compounds related to *p*-aminobenzoic acid which Woods tested, only two, novocaine and *p*-hydroxylaminobenzoic acid possessed antagonistic action comparable to *p*-aminobenzoic acid; both these compounds might be readily convertible to *p*-aminobenzoic acid by familiar biological reactions. From this evidence, Woods felt justified in suggesting that the "natural" antagonist occurring in yeast and bacterial extracts which gave reactions for an aromatic amino acid was in fact *p*-aminobenzoic acid (P.A.B.A.). He further suggested that P.A.B.A. is an essential metabolite and that "*the enzymic reaction involved in the further utilisation of P.A.B.A. is subject to competitive inhibition by sulphanilamide and that this inhibition is due to a structural*

relationship between sulphanilamide and P.A.B.A. which is the substrate for the enzymic reaction in question." Woods also suggested that the sulphonamide resistance of some organisms might be due to the ability of these organisms to synthesise sufficient P.A.B.A. to antagonise all the sulphanilamide present.

The alternative theory that sulphanilamide interferes with synthesis, rather than utilisation, of P.A.B.A. is disposed of by the existence of a competitive relationship between sulphanilamide and P.A.B.A. If synthesis, not utilisation, of P.A.B.A. were inhibited, the addition of a threshold quantity of P.A.B.A. to the medium would meet the needs of the organism and permit growth independently of the amount of sulphanilamide present; such is not the case, and increase in concentration of sulphanilamide has to be met by a corresponding increase in P.A.B.A.

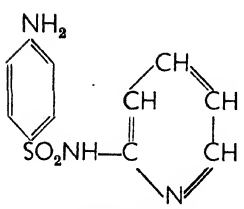
The anti-sulphonamide action of P.A.B.A. is evident *in vivo* as well as in culture media (Selbie, 1940). Mice infected with lethal amounts of *Strep. hæmolyticus* may be protected by a dose of sulphanilamide which has no protective effect if P.A.B.A. is administered simultaneously.

The suggestion as to the nature of the sulphonamide antagonist extracted from micro-organisms was confirmed by the isolation of P.A.B.A. from yeast extracts (Rubbo and Gillespie, 1940; Blanchard, 1941). P.A.B.A. was also found to exist in a bound form as a glutamic acid peptide (Ratner, Blanchard, Coburn and Green, 1944), a particularly interesting observation in view of the recent elucidation of the structure of folic acid (p. 210) (Angier *et al.*, 1946).

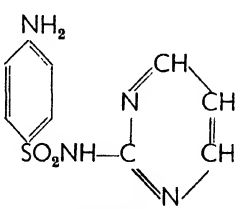
It must be emphasised that the original observations of Woods on the antagonistic action of P.A.B.A. do not constitute evidence for the metabolic role of the antagonist. Evidence for the metabolic function of P.A.B.A. had to be sought after the suggestion of its possible metabolic importance had been made. It was soon shown to be a growth factor for *Clostridium acetobutylicum* (Rubbo and Gillespie, 1940), and later for *Acetobacter suboxydans*, *Lactobacillus arabinosus*, *Corynebacterium diphtheriæ* and for certain strains of yeast. These observations naturally stimulated interest in and acceptance

of Woods' theory, but it was not possible to demonstrate the essential metabolic function of P.A.B.A. for all organisms which were sensitive to sulphonamides. Indirect evidence for the essential nature of P.A.B.A. was provided by recognition of its general distribution in animal, plant and bacterial cells. In many products, a considerable proportion was present in the " bound " form, and only released by autolysis or hydrolysis. To meet the difficulty of estimation of the very small quantity of P.A.B.A. involved, an ingenious microbiological method was introduced by Mirick (1943). A strain of soil bacillus, isolated by growing on P.A.B.A. as the only source of carbon and nitrogen, produced an enzyme specifically adapted to oxidise P.A.B.A. A particularly striking confirmation of the essential metabolic role of P.A.B.A. was obtained by isolation of an X-ray mutant strain of *Neurospora* for which P.A.B.A. was an essential growth factor. The normal strain did not require P.A.B.A., but apparently was capable of synthesising its own requirements. The mutant strain had lost this capacity, without at the same time losing the need for P.A.B.A. as a component of its metabolic system (Tatum and Beadle, 1942).

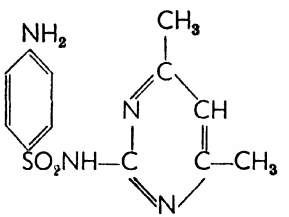
Before discussing other theories of sulphonamide action, it will be as well to summarise the agreed facts about the action of sulphonamides on living organisms. All sulphonamides



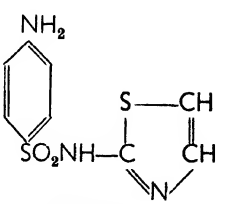
Sulphapyridine



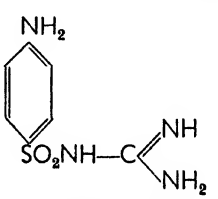
Sulphadiazine



Sulphamethazine



Sulphathiazole

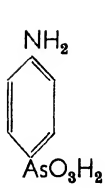


Sulphaguanidine

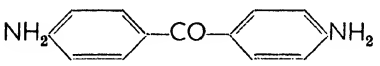
having a structure essentially similar to P.A.B.A. are antagonised by P.A.B.A. in their antibiotic effect on most living organisms. The essential structure may be defined as a free aromatic amino group in the para-position to a sulphonic acid or other acidic group. The conversion of the sulphonic acid to an amide enhances activity, particularly when the amide group derives from a suitable heterocyclic base. The formulæ of some of the clinically useful sulphonamides are given.

Compounds of related structure such as marfanil,

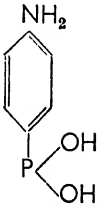
in which the primary amino group is not aromatic, may be bacteriostatic, but are not antagonised by P.A.B.A. On the other hand, aromatic amines in which the sulphonic acid radical is replaced by other acidic radicals such as AsO_3H_2, CO.Ph, PO_2H_2, or SH as in atoxyl, diaminobenzophenone, aminobenzenephosphonous acid or aminothiophenol are bacteriostatic for some organisms, and this bacteriostasis is reversed by P.A.B.A. (see Northey, 1940; Schmidt and Sesler, 1946; Kuhn, Möller, Wendt and Beinert, 1942; Kuhn, Möller and Wendt, 1943; Klotz and Morrison, 1947, for fuller discussion).



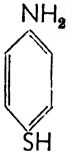
Atoxyl



4 : 4-Diaminobenzophenone

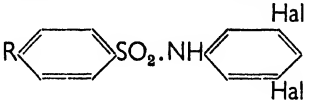


Aminobenzene-phosphonous acid



Amino-thiophenol

Halogenated sulphonamides of the general type



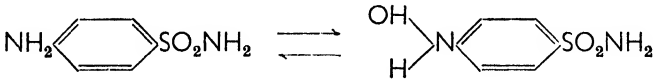
are bacteriostatic even if R is not an aromatic amino group, and are not necessarily antagonised by P.A.B.A. (Goetchius

and Lawrence, 1945; Schmidt and Sesler, 1946). It appears to be fairly certain then, that theories based on competitive inhibition will only be applicable to those sulphonamides possessing a free aromatic amino group. In this connection, it is of interest to note that organisms rendered resistant to sulphanilamide are resistant to all other sulphonamides which are normally antagonised by P.A.B.A., but are susceptible to the sulphonamides not antagonised by P.A.B.A. (see p. 259).

It is generally accepted that sulphonamides act by inhibiting the growth of micro-organisms rather than by killing directly (*i.e.* are bacteriostatic rather than bactericidal).

Sulphonamide inhibition is completely reversed by P.A.B.A. at all concentrations of sulphonamide. The amount of P.A.B.A. required to reverse the bacteriostasis is some thousand times lower than the sulphanilamide concentration. This is consistent with the theme developed in Chapter IV, p. 172, where it was suggested that natural metabolites have greater affinity for enzymes than most of their analogues because of the high specificity of enzymes for their substrates.

As evidence in favour of the Woods' theory accumulated, earlier theories as to the mode of action of sulphonamides had to be abandoned. In 1937 Mayer and Oechslin had suggested that sulphonamides owed their bacteriostatic effect to oxidation at the site of action to hydroxylaminobenzene sulphonamides (Mayer, 1937; Mayer and Oechslin, 1937).



When it was established that P.A.B.A. could antagonise completely the therapeutic (*i.e.* bacteriostatic) action of sulphonamides *in vivo*, but could not prevent the bactericidal effects of hydroxylaminobenzenesulphonamides *in vitro*, it became apparent that the theory was untenable, and that in all probability hydroxylaminobenzenesulphonamides were rapidly converted *in vivo* to aminobenzenesulphonamides. This would explain the observation that hydroxylaminobenzene-sulphonamides are much more bactericidal than sulphonamides

in vitro but no more active *in vivo*. The anti-catalase theory of Locke, Main and Mellon (1938) assumed the existence of hydroxylaminobenzenesulphonamide at the site of action as an inhibitor of catalase, the enzyme responsible for the destruction of (and protection of the cell from) any hydrogen peroxide formed during metabolism. This theory also had to be discarded because of the absence of complete competitive antagonism of hydroxylaminobenzenesulphonamide by P.A.B.A.

One theory of the relationship between sulphonamides and P.A.B.A., that of Sevag and his colleagues must, however, be considered in detail (Sevag and Shelburne, 1942*b*; Sevag, 1946). This theory is based on the premise that P.A.B.A. is not an essential metabolite but a non-toxic analogue, able to displace sulphonamides non-specifically from any enzyme surface without itself inhibiting to the same extent the normal action of the enzyme concerned. It also differs from the Woods' theory in suggesting that certain sulphonamides specifically inhibit certain respiratory enzymes to whose coenzymes they are structurally related. By inhibiting respiratory enzymes, the sulphonamides are said to deprive cells of the energy-yielding reactions, and so of the energy necessary for cell division and growth. It will be noted that this theory involves two distinct sections : (*a*) the nature of the competition between P.A.B.A. and sulphonamides, and (*b*) the type of enzyme inhibited—a point left entirely open in the Woods' theory.

Sulphonamide inhibition of bacterial respiration was found to be proportional to sulphonamide inhibition of growth (Sevag and Shelburne, 1942*a*). Further, under suitable nutrient conditions, where organisms were able to respire but not to grow, respiration was inhibited to a lesser degree by sulphonamides than was the case with growing cells. P.A.B.A. reversed sulphonamide inhibition of respiration in low concentrations (6×10^{-3} to 6×10^{-4}M), but in higher concentrations (12×10^{-3} to 35×10^{-3}M) was itself inhibitory. These results were interpreted by Sevag as indicating that a sulphonamide-sensitive respiration existed, inhibition of which caused

stoppage of growth ; also that P.A.B.A. was not an essential metabolite but simply a less toxic chemical analogue of sulphanilamide with affinity for the same enzyme systems. Evidence was available that sulphapyridine and sulphathiazole might act by interference with coenzymes I and II which are involved in respiration (Dorfman, Rice, Koser and Saunders, 1940 ; Dorfman and Koser, 1942). When the dysentery bacillus was grown in a synthetic medium deficient in nicotinamide and respiration was stimulated by addition of nicotinamide, this stimulated respiration was inhibited by sulphapyridine or sulphathiazole but not by sulphanilamide, sulphadiazine, sulphapyrazine or sulphacetamide. The inhibition was not reversed by P.A.B.A., but was prevented by nicotinamide, if it was added prior to the sulphonamide. In view of the isosterism between the pyridine and thiazole rings (Schmelkes, 1939 ; Tracy and Elderfield, 1940) the similar action of sulphapyridine and sulphathiazole was to be expected (see also p. 218).

Sevag and his colleagues carried these observations further by showing that bacterial enzymes decarboxylating pyruvic acid (carboxylases) were partially inhibited by sulphonamides, and that sulphathiazole, the sulphonamide most nearly related chemically to co-carboxylase (thiamin pyrophosphate), exerted the greatest inhibitory effect (Sevag, Shelburne and Mudd, 1942, 1945 ; Sevag, Henry and Richardson, 1945). Co-carboxylase antagonised in a competitive manner the inhibition by sulphathiazole of carboxylase activities of whole yeast, *Staph. aureus* or *Escherichia coli*. P.A.B.A. was able to reverse the inhibitory action of sulphathiazole on carboxylase activity of *S. aureus* and *E. coli*, despite the fact that in this reaction it could not possibly play the role of substrate. On the other hand, P.A.B.A. had no antagonistic effect on the inhibition of carboxylase by acetaldehyde. These results showed that P.A.B.A., in the particular case under examination, had a non-specific ability to protect an enzyme from the inhibitory effect of a sulphonamide. This non-specific antagonistic relationship between sulphanilamide and P.A.B.A. is also shown in a simpler physical system. The adsorption of

methylene blue by charcoal is inhibited by sulphanilamide; in the presence of P.A.B.A., sulphanilamide loses its inhibitory action (Eyster, 1943).

Parallelism between sulphonamide structure and degree of inhibition of respiratory enzymes through competition with coenzymes has not been extended by more recent work (Altman, 1946). Glucose-phosphate dehydrogenase (Zwischenferment) was considerably inhibited by sulphanilamide, but to a lesser extent by sulphapyridine, sulphathiazole or sulphadiazine. The enzyme could be protected by the presence of either coenzyme (triphosphopyridine nucleotide) or substrate, but in their absence, sulphanilamide formed an irreversible complex. Moreover, P.A.B.A. exerted no protective effect.

Since sulphonamides exert their therapeutic effect by preventing growth of micro-organisms, any satisfactory theory of their mode of action must be based upon results obtained from studies on bacterial growth. In general, Sevag's respiration studies were confirmed by experiments on growth, but these experiments have been criticised because of the high concentrations of sulphonamides used. A more serious difficulty is that several cases have been reported where effect of a sulphonamide on growth is not paralleled by its effect upon respiration. The inhibition of respiration of hæmolytic streptococci by sulphanilamide is no greater than the inhibition of respiration of *Staph. aureus* by the same concentration of drug, although the growth of hæmolytic streptococci is more readily inhibited than the growth of *Staph. aureus* (Wyss, Strandskov and Schmelkes, 1942). Moreover, the nicotinamide-stimulated respiration of dysentery bacillus was inhibited by sulphapyridine but not by sulphanilamide; growth was, however, inhibited by sulphanilamide as well as by sulphapyridine. The inhibition of growth by sulphapyridine or sulphanilamide was completely antagonised by P.A.B.A., but the inhibition of respiration caused by sulphapyridine was not reversed by P.A.B.A. (Dorfman, Rice, Koser and Saunders, 1940; Dorfman and Koser, 1942). Additional difficulties are raised by other observations; acetylation of sulphapyridine lowered its growth-inhibitory effect without lowering its effect

as an inhibitor of respiration. The growth of a strain of *Escherichia coli* rendered resistant to sulphonamides was inhibited to the extent of 50 per cent. by 0·0044M sulphanilamide, while in the parent strain growth was inhibited to the same extent by 0·00024M sulphanilamide; despite this difference in growth-sensitivity, the respiration of both strains was inhibited to the same extent by 0·04M sulphanilamide (Wyss, Strandskov and Schmelkes, 1942). Respiration of "P.A.B.A.-less" *Neurospora* mutants and of the normal parent strain was unaffected by all concentrations of sulphanilamide, although growth was inhibited in both cases (Tatum and Giese, 1946).

This lack of parallelism between the inhibitory actions of sulphonamides on growth and on respiration has its counterpart in the acridine series. Ferguson and Thorne (1946) found that with *Escherichia coli* the order of activity of several acridine derivatives in inhibiting growth was not the same as their order in inhibiting oxidation of various substrates. It appears that the bacteriostatic action of drugs is not dependent mainly on inhibition of oxidative energy-producing reactions; it is more likely that reactions closely connected with synthetic processes are inhibited (see p. 184). Tatum and Giese (1946) observed that germination of *Neurospora* conidia is much more sensitive to the inhibitory action of sulphanilamide than is the growth of actively-growing cultures. They suggest that in conidia, enzymes are present in low concentrations and active synthesis is required for growth; whereas in growing mycelia, the enzymes are already present in larger amounts.

Sevag's suggestion is that P.A.B.A. might be non-specific in its action and owe its sulphonamide-antagonistic action to its ability to displace sulphonamides from surfaces upon which they were adsorbed, without itself being an essential metabolite or playing any part in essential enzymic processes of the cell. While this point of view must be regarded as quite legitimate, there is absolutely no doubt that P.A.B.A. does play some vital function in growth and cellular metabolism. There is evidence also that in some cases sulphonamide-

resistant organisms do synthesise P.A.B.A. in greater amounts than their parent strains (Landy and Gerstung, 1944 ; Landy, Larkum, Oswald and Streightoff, 1943). P.A.B.A. is also unique in that it is capable of antagonising completely the bacteriostatic effect of many sulphonamides over a wide range of concentration ; none of the respiratory coenzymes has a comparable effect. The P.A.B.A. reversal of inhibition of carboxylase activity caused by sulphathiazole is not invariably complete (Sevag, Henry and Richardson, 1945). The observation of Sevag that P.A.B.A. can itself act as an enzyme inhibitor and a growth inhibitor in high concentrations is not inconsistent with the known facts of enzymology, since in many cases when substrate concentration is increased beyond a certain point the substrate acts as an inhibitor.

Other metabolite analogues such as pyrithiamine, glucoascorbic acid, etc. (see p. 220), when fed to animals produce symptoms which can be ascribed to interference with the normal function of the related metabolite. Sulphathiazole does not produce symptoms of co-carboxylase deficiency in higher animals. The usual finding after feeding of sulphonamides to laboratory animals is inhibition of bacterial growth in the intestine with a resulting deficiency of factors believed to be obtained by animals from bacterial synthesis in the intestine, such as biotin and vitamin K (Daft and Sebrell, 1945) ; deficiency symptoms are relieved by feeding of these factors. The only clearly defined toxic reactions to large doses of sulphonamides in man are granulocytopenia and leucopenia. It is suggestive that folic acid seems to be particularly concerned with the reproduction of bone marrow cells, and that sulphonamides, which may prevent synthesis of folic acid, cause failure of bone marrow cell reproduction. Evidence for a relationship between sulphonamide inhibition and folic acid synthesis is discussed more fully in succeeding sections.

The evidence discussed so far would seem to leave the original suggestion of Woods that sulphonamides act in virtue of their similarity to an essential metabolite, viz. P.A.B.A., as the only satisfactory theory for the mode of action of these

drugs. Some sulphonamides do inhibit respiratory enzymes, but this may be a secondary rather than a primary action. *There is no reason to suppose that bacteriostatic agents always act on a single stage in the chain of enzymic reactions involved in cell metabolism; some sulphonamides may act on one stage only, others may be capable of inhibiting several.* The evidence collected by a number of investigators on the quantitative relationship between sulphonamide inhibition and P.A.B.A. antagonism does suggest, however, that all sulphonamides possessing a free primary aromatic amino group are caught up in cellular metabolism at, at least, one common point. Bradbury and Jordan (1942), from a study of the electrokinetic mobility of suspensions of *Escherichia coli* in dilute solutions of either sulphonamides or P.A.B.A., concluded that all the bacteriostatic sulphonamides and P.A.B.A. affected electrokinetic mobility in the same way, and were probably adsorbed in the same way by the cell; whereas inactive related compounds such as aniline and *m*-aminobenzene sulphonamide behaved quite differently. Fox and Rose (1942) found that although the bacteriostatic potency of various sulphonamides varied over a wide range, the amount of P.A.B.A. required to antagonise the minimal effective concentration (M.E.C.) of drug was constant (see Table 13).

TABLE 13

Minimum effective concentration of sulphonamides and amount of P.A.B.A. required to antagonise this concentration
(Fox and Rose, 1942)

	M.E.C. ($M \times 10^{-6}$)	Amount P.A.B.A. for Reversal of Bacteriostasis ($M \times 10^{-6}$).
Sulphanilamide	2500	0·5
Sulphapyridine	20	0·5
Sulphathiazole	4	0·5
Sulphadiazine	4	0·5

It is difficult to interpret such observations in any other light than that suggested above, namely, the identity of one primary site of action of all sulphonamides antagonised by P.A.B.A. Such quantitative studies, however, do not help to elucidate the finer point as to the nature of the system inhibited, although they have been extremely useful in

correlating structure with antibiotic action among sulphonamides (see Chapter VII). It is not possible at present to do more than guess at the nature of the enzyme system actually involved, because as pointed out in an earlier chapter, we are largely ignorant as yet concerning the synthetic processes going on in living cells.

Sulphonamide antagonists other than P.A.B.A.

Further complexity has been added to the problem of sulphonamide action by the array of so-called secondary sulphonamide antagonists. In discussing these, it must be emphasised that were a single enzymic reaction of a living cell inhibited by a drug, the results would extend along a whole chain of inter-related enzyme systems. This would result in various aberrations of normal metabolism, each subject to further modification or restoration by addition of intermediate metabolites to the nutritive medium.

At least four general types of sulphonamide antagonist may be listed :—

(1) Essential metabolites which are displaced or replaced by sulphonamides, *e.g.* P.A.B.A.

(2) Substances which, when added to a nutritionally poor medium, increase rate of growth and so mask sulphonamide inhibition in a non-specific way; glucose may act in this way in suitable media.

(3) Compounds which combine with sulphonamides and render them unavailable for bacteriostasis, *e.g.* proteins, urethane.

(4) Metabolites normally synthesised by the cell in reactions secondary to, and dependent upon, a primary reaction which is inhibited by sulphonamides, *e.g.* methionine.

The first type of antagonist we have already discussed; to the second group belong all those substances which cannot be shown to act in any specific way. When drug antagonism is observed, the antagonist can be placed in this category

until it has been shown by experiment to belong elsewhere. It is well known that sulphonamides are more effective bacteriostatic agents the more unfavourable the cellular environment. It is often necessary to make careful quantitative experiments before it is possible to demonstrate that an apparently specific antagonism is due to non-specific growth stimulation. Johnson, Eyring and Kearns (1943), using luminescent bacteria, studied the effect of various media on sulphonamide inhibition of luminescence and concluded that peptone and glucose and possibly arginine, hypoxanthine and serum acted by stimulating luminescence rather than by antagonising sulphonamide. Green and Bielschowsky (1942), in their study of the sulphonamide-antagonistic "*p*" factor, concluded that it contained, in addition to the specific antagonist P.A.B.A., an unknown non-specific growth stimulant. The apparent sulphonamide antagonism shown by nicotinamide, nicotinic acid or cozymase for *Staph. aureus* may have been due to a non-specific growth stimulatory effect; in cultures of *Escherichia coli* where these substances failed to stimulate growth, they also failed to influence the bacteriostatic action of sulphonamides (Wood and Austrian, 1942). Peptone and yeast extracts exercised some antagonistic effect by growth stimulation of *E. coli* (Rantz and Kirby, 1944). Even physical factors such as temperature may alter significantly the degree of sulphonamide inhibition (Johnson, Eyring and Kearns, 1943).

The third type of antagonism which we have listed, combination of antagonist with drug, may occasionally be obvious from inspection of the chemical nature of drug and antagonist, but more subtle grades of combination antagonism also exist. Kimmig and Weselmann (1941) showed by cataphoretic methods that all sulphonamides were adsorbed by serum albumin but not by serum globulin. Davis confirmed that proteins combine with sulphonamides and partially prevent their dialysis through cellophane membranes. In normal human plasma, sulphanilamide is bound to the extent of 20 per cent., sulphapyridine 40 per cent., sulphadiazine 55 per cent. and sulphathiazole 75 per cent.; however, the

free *p*-amino group is not essential for protein binding although it is known to be essential for bacteriostatic action (Davis and Wood, 1942; Davis, 1942, 1943).

Urethane and the related barbiturate nembutal exert a marked sulphonamide antagonism which is difficult to account for on any structural analogue basis. The action is not competitive and is only functional at low concentration (McIlwain, 1942*a*). Johnson and his colleagues have made a quantitative study of the urethane antagonism of sulphonamide inhibition of luminescence in luminescent bacteria, and concluded that, in this system, urethane formed with sulphanilamide an inactive complex (Johnson, Eyring and Kearns, 1943).

The fourth important group of sulphonamide antagonists other than P.A.B.A. includes amino acids, thymine and purines. Methionine $CH_3.S.CH_2.CH_2.CH(NH_2).COOH$ was recognised as a sulphonamide antagonist by Harris and Kohn (1941), also by Bliss and Long (1941). Besides being an essential amino acid for higher animals, methionine is an essential constituent of media for certain strains of diphtheria bacillus (Mueller, 1935), for some streptococci (Smiley, Niven and Sherman, 1943), for *Clostridium sporogenes* (Fildes and Richardson, 1935) and for some strains of lactic acid bacteria (Dunn *et al.*, 1947). It does not seem to act as a sulphonamide antagonist by virtue of its growth-promoting properties; other amino acids such as cystine or tyrosine, which on the same media have greater growth-stimulating effect, have no sulphonamide-antagonistic properties. Reversal of sulphanilamide bacteriostasis by methionine can only be achieved at low concentration of sulphanilamide, so that methionine cannot be a competitive antagonist displacing sulphanilamide from its site of action. Other amino acids, such as glycine, *d.l*-serine and *d.l*-allothreonine, do not antagonise sulphanilamide bacteriostasis by themselves, but each enhances the antagonistic action of methionine. Guanine and xanthine also enhance the methionine antagonism of sulphanilamide, but, in the absence of methionine, both are without antagonistic action and indeed enhance sulphonamide bacteriostasis.

There would appear to be some unknown relationship between P.A.B.A., methionine and purine metabolism.

Attempts to elucidate the metabolic function of methionine in *E. coli* have not given much information. It is not oxidised, decarboxylated, deaminated or hydrolysed by washed-suspensions of the organism, and cannot replace ammonium ion in the basal medium. It only becomes a growth factor when the organism is repeatedly subcultured in a medium containing sulphanilamide and methionine (Kohn and Harris, 1942). These observations are difficult to interpret, particularly as results with other species of organism under other experimental conditions do not always agree. Much of the hesitation in accepting the simple and straightforward suggestion of Woods as to the relationship between P.A.B.A. and sulphonamides, can probably be ascribed to confusion introduced in attempts to explain these observations.

Kohn (1943) has suggested that his observations can be explained by an extension of Woods' theory. When sulphonamides interfere with P.A.B.A. metabolism, they are said to prevent formation of metabolites whose synthesis is subsequent to, and dependent upon, proper functioning of the P.A.B.A. system. He visualises the process in the case of *Escherichia coli* according to the following scheme (Fig. 21) :—

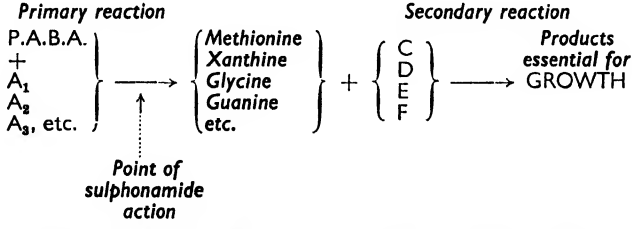


FIG. 21.—Mode of action of sulphonamides (Kohn, 1943).

The primary reactions in which P.A.B.A. takes part, together with various substances of unknown nature, can be inhibited by sulphonamides, some reactions being more sensitive than others. The cell is regarded as dependent upon the proper functioning of these reactions for its supply of secondary metabolites such as methionine. These in turn are

supposed to be necessary for secondary reactions involving formation of still further metabolites, in the absence of which growth is not possible. If only one of the primary reactions is inhibited, say that responsible for methionine supply, then growth can be restored by supplementing the nutrient medium with methionine.

These views have gained support from various observations. With *Escherichia coli*, sulphonamide bacteriostasis is directed against growth rather than against respiration. Ethionine, $C_2H_5.S.CH_2.CH_2.CH(NH_2).COOH$, the higher homologue of methionine, inhibits growth of *E. coli* on a synthetic medium; its effect is completely reversed by one-tenth of its concentration of methionine (Harris and Kohn, 1941). Methoxinine, $CH_3.O.CH_2.CH_2.CH(NH_2).COOH$, the oxygen analogue of methionine, was also growth inhibitory for *E. coli*, the growth-inhibitory effect being prevented by *l*-methionine but not by the *d*-isomer (Roblin, Lampen, English, Cole and Vaughan, 1945). Purines in high concentration were able to replace P.A.B.A. in the nutrition of *Clostridium acetobutylicum* (Housewright and Koser, 1944); with *Acetobacter suboxydans*, which also requires P.A.B.A. as a growth factor, addition of purines to the growth medium increased growth at low P.A.B.A. concentrations, but not at high concentrations (Landy and Streightoff, 1943).

Strong support of Kohn's theory also comes from a study of the nutritive requirements of a strain of *Escherichia coli* produced by exposure of a normal strain to X-rays. The variant strain required P.A.B.A. as a growth factor, but growth response to P.A.B.A. was enhanced by the presence of amino acids, of which the most important was methionine. In the absence of amino acids, purines delayed growth, but in their presence purines promoted growth. In the presence of both amino acids and purines, the P.A.B.A. requirements fell from $0{\cdot}0012\gamma$ per c.c. to $0{\cdot}00011\gamma$ per c.c. Thymine was practically inactive alone, but in the presence of purines it supported slow growth. A medium containing thymine, purines and amino acids enabled the organism to dispense altogether with P.A.B.A. In this medium both the parent

strain and the variant were highly resistant to sulphonamides (Lampen, Roepke and Jones, 1946).

The amount of sulphanilamide required for maximum growth inhibition of *Lactobacillus arabinosus* in the presence of adenine or other purines was ten times that required in the absence of purines (Shive and Roberts, 1946). Methionine was without effect as a sulphanilamide antagonist for this organism, either in the presence or absence of purines. With *Escherichia coli*, on the other hand, methionine by itself showed some antagonism, while purines had no effect; a mixture of methionine and xanthine or guanine raised the sulphonamide concentration necessary for inhibition by a factor of ten. The P.A.B.A. analogue, 4-amino-2-chlorobenzoic acid, acted as a growth inhibitor antagonised competitively by P.A.B.A., but was inactive in the presence of methionine.

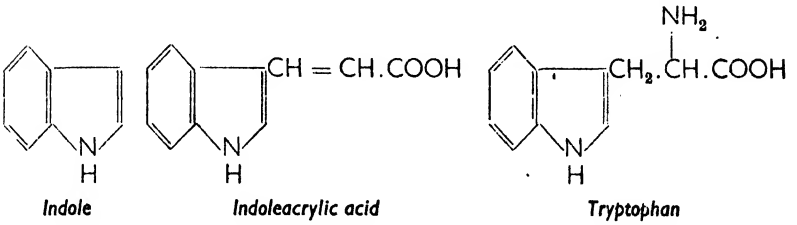
These results all suggest that P.A.B.A. is part of a cellular mechanism for the synthesis of purines, certain amino acids and possibly pyrimidines, and that the synthesis of these essential metabolites is prevented by sulphonamides.

Essential metabolites as non-competitive drug antagonists

The antagonism of sulphonamides by the combined action of amino acids, purines and pyrimidines is non-competitive, and probably takes place through replacement of an intracellular source of metabolite by an extracellular supply. Other examples of non-competitive reversal of growth inhibition which have been recognised can also be explained on this basis.

Fildes (1941, 1945) showed that tryptophan is an essential metabolite for many bacteria, some of which are capable of synthesising their requirements from indole. Indoleacrylic acid is an effective inhibitor of growth in an indole medium, but the inhibition can be completely reversed by addition to the medium of a small quantity of tryptophan. The relationship is non-competitive since the same amount of tryptophan is required to restore growth irrespective of the concentration of indoleacrylic acid, a fivefold increase in indoleacrylic acid necessitating no further increase in tryptophan. Indoleacrylic acid was shown to prevent the synthesis of tryptophan which

probably occurs by condensation of indole with serine. Addition of preformed tryptophan to the medium allows an essential enzymic process related to growth to proceed, despite



Indole *Indoleacrylic acid* *Tryptophan*

the inability of the organism to synthesise tryptophan. Various methyl derivatives of both indole and tryptophan inhibit growth of *Eberthella typhosa*, and here again the effect is reversed non-competitively by tryptophan. Indole itself antagonises in a competitive manner (Fildes and Rydon, 1947).

This example differs somewhat from the sulphonamide methionine relationship where antagonism is competitive over a small range and incomplete at higher sulphonamide concentrations. It is closely analogous to the effect observed by Lampen (p. 205) with an " aminobenzoic-less " mutant of *Escherichia coli* whose growth in absence of P.A.B.A. was completely restored by a mixture of thymine, purines and amino acids and was then no longer susceptible to inhibition by sulphonamides.

A further example of non-competitive antagonism reported by Beerstecher and Shive (1947 *a* and *b*) seems to have a similar basis. Tyrosine had no effect on the bacteriostatic action of β-hydroxyphenylalanine which inhibited *Escherichia coli* by competition with phenylalanine, it did reverse the bacteriostatic action of thienylalanine, which also competed with phenylalanine. Apparently, phenylalanine is necessary for at least two different types of reaction in *E. coli*; one of these is inhibited by β-hydroxyphenylalanine and produces unknown products; the other is inhibited by thienylalanine and is normally required by the cell for synthesis of tyrosine. If free tyrosine is supplied in the medium, the organism no longer requires an enzyme system for synthesis of tyrosine,

and is accordingly no longer susceptible to inhibition by thienylalanine (Fig. 22). It should be noted that this scheme is based on the assumption that phenylalanine is a precursor of tyrosine for *E. coli*; this does not agree with work of Simmonds, Tatum and Fruton (1947) on two mutant strains of *E. coli*. These workers suggest that the mutants do not synthesise tyrosine from phenylalanine.

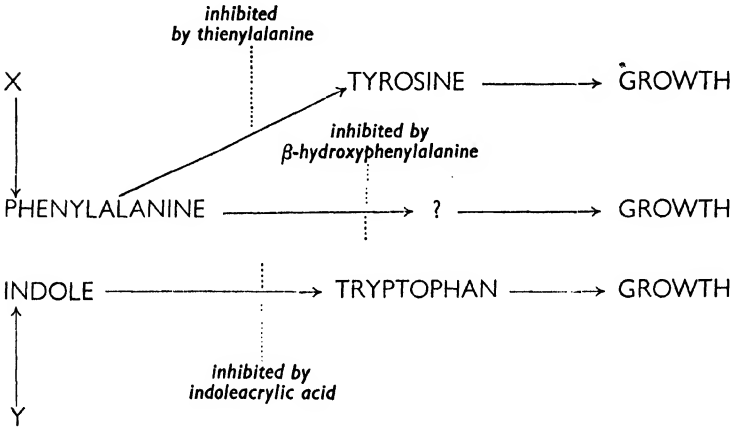
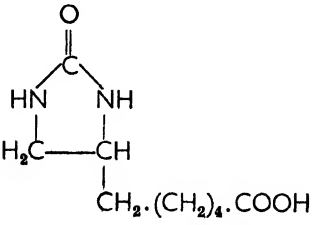
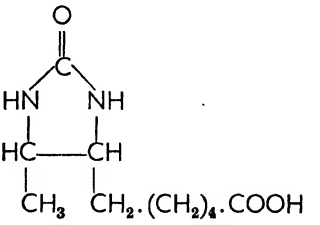


FIG. 22.—Non-competitive antagonism in amino acid utilisation.

Another example of antagonism by two closely-related metabolites, one a competitive and the other a non-competitive antagonist, may be cited from the field of biotin chemistry (*cf.* p. 142). Imidazolidonecaproic acid (I) is growth-inhibitory for several micro-organisms, and is antagonised competitively by the biotin precursor desthiobiotin (II).



I



II

Presumably imidazolidonecaproic acid competes with desthiobiotin and prevents its conversion to biotin. Such an interpretation is confirmed by the fact that biotin also antagonises imidazolidonecaproic acid, but in a non-competitive manner (Rogers and Shive, 1947 ; Dittmer and du Vigneaud, 1947).

Methionine sulphoxide has been investigated as a growth-inhibitory analogue of glutamic acid. The sulphoxide apparently prevents amidation of glutamic acid to glutamine since it is antagonised non-competitively by glutamine (Waelsch, Owades, Miller and Borek, 1946). Support for this view is provided by the report of Elliott and Gale (1948) that the enzyme system of *Staph. aureus* which converts glutamic acid to glutamine is inhibited by methionine sulphoxide, inhibition being competitive with respect to glutamic acid.

$CH_3.SO.CH_2.CH_2.CH(NH_2).COOH$
Methionine sulphoxide

$HO.CO.CH_2.CH_2.CH(NH_2).COOH$
Glutamic acid

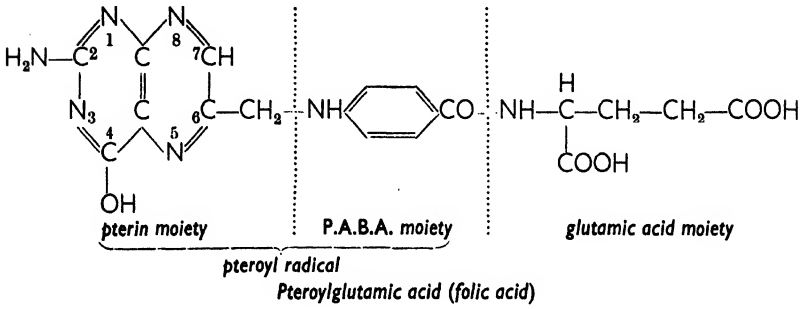
$NH_2.CO.CH_2.CH_2.CH(NH_2).COOH$
Glutamine

Folic acid and the site of sulphonamide inhibition

The above examples make it apparent that antagonism of a bacteriostatic substance may be achieved either by adding a metabolite which can compete with the drug at a reactive centre, or alternatively by supplying the product which cannot be synthesised in the presence of drug. In the first case only, the relationship will have the characteristics of competitive inhibition.

If these principles are borne in mind, recent studies of the relationship of pteroylglutamic acid (folic acid) to sulphonamide inhibition indicate one site of action of sulphonamides (Lampen and Jones, 1946 *a* and *b*). We have already noted (Chapter III) that growth of some organisms in the presence of P.A.B.A. is accompanied by folic acid synthesis. Miller (1944) noted that, when bacteria were grown in sub-inhibitory concentrations of sulphonamide, the amount of folic acid synthesised was markedly reduced. Disclosure of the chemical

structure of folic acid as pteroylglutamic acid (formula below) suggested that sulphonamides might displace P.A.B.A. from the enzyme surface on which the pteroylglutamic acid molecule was fitted together.



Lampen and Jones found that growth of *Strep. fæcalis* and *Lactobacillus casei* in the presence of pteroylglutamic acid was insensitive to sulphonamides. Strains which were able to synthesise pteroylglutamic acid were found to be sensitive to sulphonamides under conditions where they were forced to synthesise this essential metabolite, but insensitive when it was supplied in the medium. A competitive inhibition occurred between sulphadiazine and P.A.B.A. or between sulphadiazine and *p*-aminobenzoylglutamic acid, but the antagonism of sulphadiazine by pteroylglutamic acid was non-competitive. Thymine also antagonised in a non-competitive manner. The same relationship was found with other sulphonamides and indicates that sulphonamides compete, in these organisms, with P.A.B.A. for an enzymic mechanism involved in the conversion of P.A.B.A. to pteroylglutamic acid.

These results, taken in conjunction with the results on amino acid and purine antagonism of sulphonamides, and with the demonstration that amino acids, purines and pyrimidines can replace P.A.B.A. in the growth medium of "P.A.B.A.-exacting" organisms, suggest that Kohn's scheme (Fig. 21) may be very tentatively extended as follows (Fig. 23).

At present the exact relationship between pteroylglutamic acid and amino acid, purine and pyrimidine synthesis is obscure. Although pteroylglutamic acid antagonises the

bacteriostatic action of sulphonamides in several organisms, it cannot replace P.A.B.A. as a growth factor for *Lactobacillus arabinosus* (Sarett, 1947) or for the " aminobenzoic acid-less "

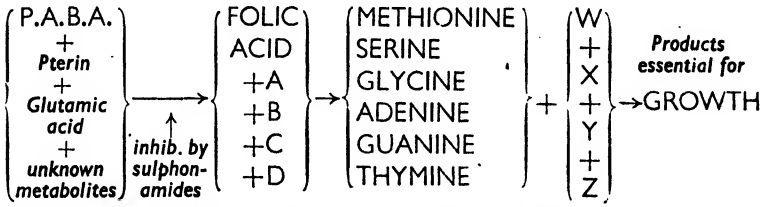


FIG. 23.—Mode of action of sulphonamides.

mutant of *Escherichia coli* studied by Lampen, Roepke and Jones (1946). This probably means that P.A.B.A. has several functions besides pteroylglutamic acid synthesis (*cf.* Chapter III).

Detailed study of pteroylglutamic acid analogues as growth inhibitors has provided confirmatory evidence for the mode of action of sulphonamides. 7-Methylfolic acid, the homologue of pteroylglutamic acid with an additional methyl group at position 7 in the pterin ring, acts as a folic acid displacing agent (Martin, Tolman and Moss, 1947 *a* and *b*). With *Staph. aureus*, the growth-inhibitory action of 7-methylfolic acid was counteracted by P.A.B.A., by pteroylglutamic acid and by pteroic acid or even by sulphathiazole, but not by glutamic acid or by *p*-aminobenzoylglutamic acid. The inhibitory action of sulphathiazole against this strain of staphylococcus was antagonised by P.A.B.A., or by pteroic acid but not by pteroylglutamic acid. This last observation is unexpected. Martin, Tolman and Moss (1947*b*) suggest that pteroic acid and not pteroylglutamic acid is involved in staphylococcus metabolism; this conclusion requires further support before it can be considered as established. A group of synthetic 2 : 4-diaminopteridines has also been tested for growth-inhibitory action and as pteroylglutamic acid displacing agents against several micro-organisms; some possessed antibacterial activity which was antagonised competitively by pteroylglutamic acid (Daniel, Norris, Scott and Heuser, 1947; Daniel and Norris, 1947). We have already seen that sulphathiazole

reduced synthesis of pteroylglutamic acid. Diaminopteridines enhanced sulphonamide bacteriostasis in organisms which normally synthesise pteroylglutamic acid.

Two further links in the chain of evidence relating sulphonamide inhibition with failure to synthesise pterins and purines may be mentioned. It has been known for some time (Fox, 1942; Stetten and Fox, 1945) that an aromatic amine accumulates in the medium when *Escherichia coli* is grown in a synthetic medium containing salts, amino acids, glucose and sulphonamides. In the presence of sufficient P.A.B.A. to block sulphonamide action the amine is not formed. Stetten and Fox suggested that the amine might be an intermediate in some metabolic reaction which was blocked by sulphonamide. The amine has now been identified as 5-amino-4-imidazole-carboxyamide, a possible precursor of purines (Shive *et al.*, 1947), and has been shown to be formed in amounts directly proportional to the glycine content of the medium (Ravel, Eakin and Shive, 1948).

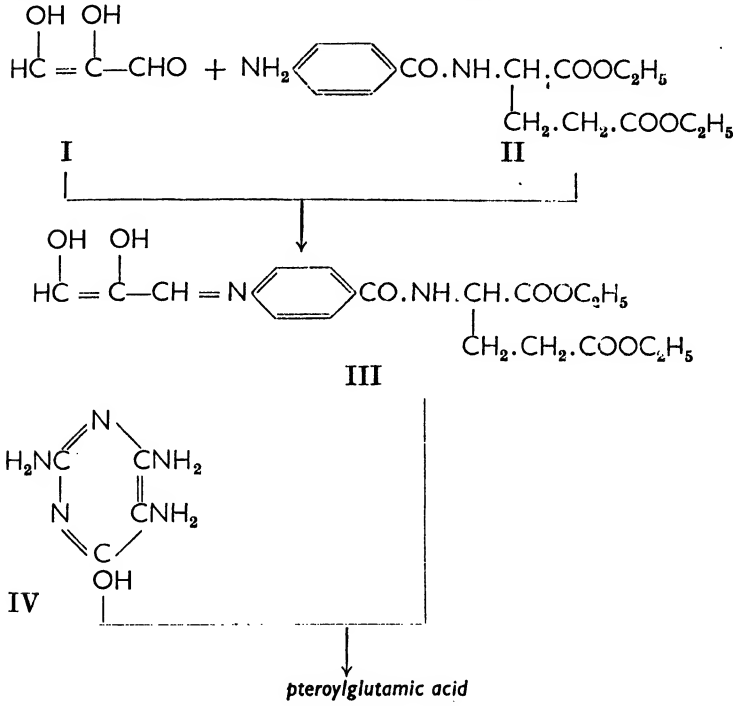
A second line of evidence derives from the suggestion that sulphonamides prevent bacterial growth by displacing P.A.B.A. from combination with glucoreductone (O'Meara, McNally and Nelson, 1947). Forrest and Walker (1948 *a* and *b*) have found that under suitable conditions condensation between triamino-hydroxypyrimidine and glucose gives rise to a pterin with an absorption spectrum essentially the same as that given by the pteroyl radical of pteroylglutamic acid; moreover, glucoreductone (I below) condenses readily with the ester of *p*-aminobenzoylglutamic acid (II) to give III. This condensation product combines readily with 2 : 4 : 5-triamino-6-hydroxypyrimidine (IV) to give pteroylglutamic acid (Angier *et al.*, 1948).

Essentially similar results have been obtained independently by Forrest and Walker (unpub.).

These results indicate that Kohn's scheme, Fig. 23, might be still further modified, the term pterin being replaced by triaminohydroxypyrimidine+glucoreductone.

From all these lines of evidence, we may sum up the present state of knowledge regarding the mode of action of sulphon-

amides, by suggesting that the primary reason for the effectiveness of sulphonamides in preventing growth of certain pathogenic bacteria resides in the need of those bacteria to synthesise



pteroylglutamic acid or a similar compound. This makes it apparent, also, why sulphonamides do not inhibit effectively all bacteria. An organism which does not carry out this synthesis, but depends instead on its environment for a supply of folic acid, will not be inhibited to the same extent by sulphonamides. It should be kept in mind, however, that any sulphonamide may have several secondary sites of inhibitory action which become obvious in those organisms which do not synthesise folic acid.

Antagonists and sulphonamide therapy

Besides their interest as pointers to the mode of drug action, sulphonamide antagonists are of considerable practical importance, and account for differences observed between the

in-vivo and *in-vitro* effectiveness of sulphonamides as bacteriostatic agents. Because of the presence of antagonists such as protein, amino acids and P.A.B.A. in body tissues and fluids, a sulphonamide which may prevent bacterial growth in culture media may be of no use therapeutically. For example, *Clostridium sordelli*, which is strongly inhibited by sulphonamides *in vitro*, cannot be controlled in animal infections (Reed, Orr and Reed, 1944).

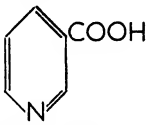
Other metabolite analogues

The amount of space devoted to a discussion of competitive inhibition by sulphonamides, besides being a tribute to the importance of sulphonamide drugs, is an illustration of the valuable stimulus provided by study of competitive inhibition to general theories of drug action. In searching for the perfect chemotherapeutic remedy for any disease, such factors as absorption, excretion, toxicity and distribution in the host must be considered, but before systematic improvement becomes possible, variables must be eliminated and the effect of structure on each factor studied separately.

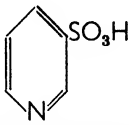
The information provided by the P.A.B.A. antagonism of sulphonamides has turned much chemotherapeutic research from brilliant exploitation of chance observations to purposeful development of growth-inhibitory metabolite analogues. For the present, this new guidance of the trend of research may have the anomalous result that, in relation to the effort expended, fewer clinically useful drugs will be added to the pharmacopœa ; but the body of information collected on the way will have greatly widened the perspective and has already begun to provide a biochemical foundation for the study of drug action.

In discussing the metabolic requirements of micro-organisms, we gave a number of examples of the substitution of an essential metabolite by a compound of closely related chemical structure, and noted how growth-promoting activity would fall as the substitute became less suitable for biological conversion to the parent structure. The recognition that substitution of the carboxyl group of P.A.B.A. by a sulphon-

amide group produced a bacteriostatic compound stimulated synthesis of other metabolite analogues in which similar structural change was made. Nicotinic acid or nicotinamide is essential for the growth of various micro-organisms and, as already noted, cannot be replaced by other pyridine derivatives not substituted in the 3-position (Chapter III). Pyridine-3-carboxylic acid esters can replace nicotinic acid to some extent, probably because they can be converted biologically to the free acid (Dorfman, Koser, Reames, Swingle and Saunders, 1939). Pyridine-3-sulphonic acid, instead of supporting growth, acts as a growth inhibitor for some organisms and its growth-inhibitory action is reversed by nicotinic acid (McIlwain, 1940).



Nicotinic acid

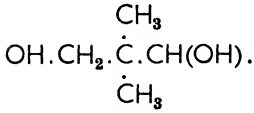


Pyridine-3-sulphonic acid

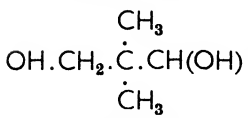
The narrow line between growth inhibition and growth promotion must be emphasised here. Organisms which do not require nicotinic acid for growth were not inhibited by the related sulphonic acid ; some micro-organisms, such as *Proteus vulgaris*, which were inhibited by pyridine sulphonic acid when nicotinic acid was the growth factor were not inhibited when nicotinic acid was replaced by nicotinamide. Other organisms actually utilised pyridine-3-sulphonic acid as a growth factor (Lwoff and Querido, 1939). With pyridine-3-sulphonic acid amide, a somewhat similar type of action was observed. This compound was bacteriostatic for organisms requiring nicotinamide as a growth factor, but had little effect even at high concentrations on organisms able to synthesise their own nicotinamide requirements. The inhibitory action was antagonised by nicotinamide.

Further extension of the idea of replacing a carboxyl group by a sulphonic acid group followed ; pantoyl-taurine, the sulphonic acid analogue of pantothenic acid, was found to be bacteriostatic for those organisms which required pantothenic acid as a growth factor (Snell, 1941 ; Kuhn, Wieland

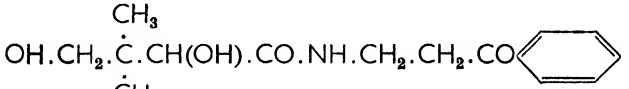
and Möller, 1941; McIlwain, 1942*b*). The bacteriostatic action was competitively reversed by pantothenic acid.



Pantothenic acid



Pantoyl-taurine



Phenylpantothenone

The close relationship of bacteriostatic action to the metabolic character of the cell is further emphasised by the observation that in the case of organisms which require for growth only one " half " of the pantothenic acid molecule in the medium, a structural analogue of that half may inhibit growth. Thus, β-aminobutyric acid and *iso*serine inhibited the growth of yeast induced by β-alanine (Nielsen and Johansen, (1943); taurine inhibited growth of *Acetobacter suboxydans* and *Clostridium septicum* more in the presence of the pantoyl moiety than in the presence of intact pantothenate, and the inhibition was reversed by β-alanine (Sarett and Cheldelin, 1945; Ryan, Schneider and Ballentine, 1947).

Pantoyl-taurine has been tried as a chemotherapeutic remedy in animal infections and exerted some protective action against streptococcal infection, but the rapid rate of excretion and the presence in blood of the natural antagonist (pantothenic acid) rendered the compound relatively ineffective (McIlwain and Hawking, 1943). Systematic variation of structure has since led to other analogues with much greater bacteriostatic action (Shive and Snell, 1945; Snell and Shive, 1945; Woolley and Collyer, 1945; Mead, Rapport, Senear, Maynard and Koepfli, 1946; Snell, 1946). One of these,

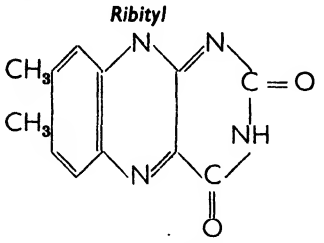
phenylpantothenone (formula above), is particularly interesting, since it inhibits growth of organisms which do not require added pantothenic acid as well as those requiring this metabolite, but its growth-inhibitory effect is reversed by pantothenic acid only in those organisms for which pantothenic acid is a growth factor.

Pantothenic acid analogues have been used with some success in experimental malaria infections (Mead, Rapport, Senear, Maynard and Koepfli, 1946; Brackett, Waletzky and Baker, 1946; Senear, Rapport and Koepfli, 1947). Pantothenic acid is probably a growth factor for the parasite, since sporozoite-induced infections in chicks were slower in developing, and less severe, in birds maintained on a pantothenic acid deficient diet. Most of the drugs which were active, also caused pantothenic acid deficiency in the chick, but some were apparently distributed so that they preferentially inhibited parasite growth. Exoerythrocitic infections were not inhibited in the same way, probably because of the higher concentration of pantothenic acid in tissues than in blood.

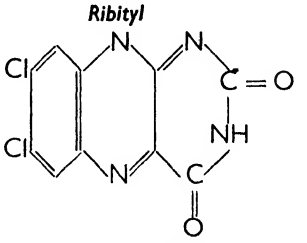
As already noted, riboflavin plays an important role as a component of many enzyme systems, and is an essential metabolite for many micro-organisms. Various synthetic analogues were found to possess reduced growth-promoting action (see Chapter III); others, such as dichloroflavin, were growth-inhibitory for those bacteria which cannot synthesise their own supply of riboflavin (Kuhn, Weygand and Möller, 1943). A phenazine analogue, 2 : 4-diamino-6 : 7-dimethyl-9-ribityl-9 : 10-dihydrophenazine has also been found to inhibit growth of *Lactobacillus casei* (Woolley, 1944*b*); while a "riboflavin-less" mutant of *Neurospora* was inhibited by both lumichrome and lumiflavin (Mitchell and Houlahan, 1946). In all these cases, the inhibition was reversed by addition of riboflavin to the growth medium (Formulæ, p. 218).

We have seen that taurine can antagonise β-alanine in the synthesis of the pantothenic acid molecule. Sulphonic acid analogues of α-amino acids also antagonise the natural amino acids (McIlwain, 1941*b*). Cysteic acid, the analogue of aspartic acid where the ω-carboxyl group is replaced by

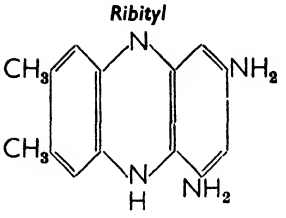
sulphonic acid, is toxic to various micro-organisms and is antagonised competitively by aspartic acid (Ravel and Shive,



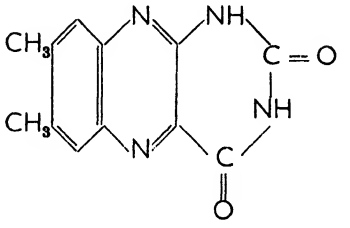
Riboflavin



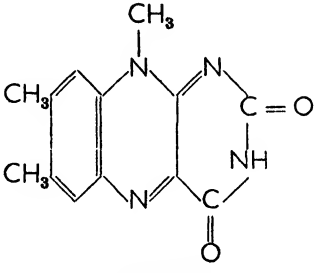
Dichloroflavin



2 : 4-Diamino-6 : 7-dimethyl--9-ribityl-9 : 10-dihydrophenazine



Lumichrome

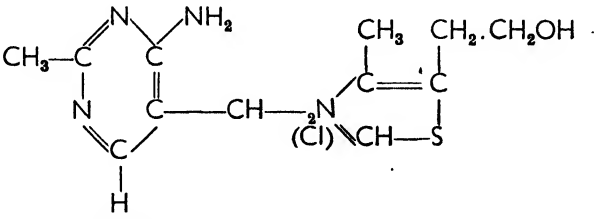


Lumiflavin

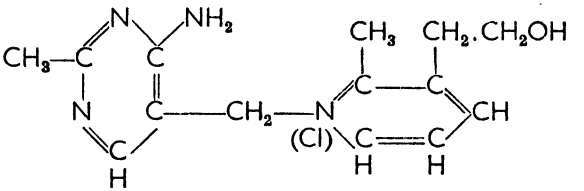
1946). In *Escherichia coli*, one reaction prevented by cysteic acid is thought to be decarboxylation of aspartic acid to β-alanine, since toxicity is not apparent in the presence of pantothenic acid or β-alanine.

The relationship between growth requirements and inhibition by a growth-factor analogue has been particularly fully worked out for the thiamin analogue pyrithiamin, in which the thiazole ring of thiamin is replaced by a pyridine ring (Tracy and Elderfield, 1940). Many organisms which

cannot synthesise thiamin, and require the intact thiamin molecule are prevented from growth by low concentrations of pyrithiamin, but organisms which can synthesise the



Thiamin



Pyrithiamin

complete thiamin molecule are highly resistant to growth inhibition (Woolley and White, 1943*b*; Wyss, 1943). It will be remembered that some micro-organisms can synthesise the complete thiamin molecule if supplied with both the pyrimidine and the thiazole portions; others have greater synthetic powers and can synthesise one half if supplied with the other half. In the case of organisms which require only the pyrimidine half, the growth-inhibitory action of pyrithiamin can be reversed by addition of the pyrimidine to the medium.

Robbins (1941) found that three fungi differed in their response to pyrithiamin in accordance with their differing growth requirements. *Phytophthora cinnamomi*, which requires the intact thiamin molecule, could not grow in the presence of low concentrations of pyrithiamin; *Phycomyces blakesleeanus*, which could grow on a mixture of thiazole and pyrimidine, was able to split pyrithiamin and use the pyrimidine half if supplied with the thiazole half; *Pythiomorpha gonapodioides*, which can synthesise the thiazole half, was able to grow on pyrithiamin by using it as a source of pyrimidine. In *Neurospora*, on the other hand, there is no relationship

between capacity for thiamin synthesis and ease of inhibition by pyrithiamin (Tatum and Bell, 1946).

Pyrithiamin cannot be used as a chemotherapeutic drug, since at blood concentrations which are non-toxic to experimental animals, the drug is not bacteriostatic (Wyss, 1943). As in the case of pantothenic acid analogues, this difficulty is related to the fact that many bacterial growth factors are also essential nutrients for higher animals. During evolution, much of the enzymic make-up of life has remained essentially unchanged, and the same substances are necessary throughout the non-photosynthetic living world for the elaboration of new enzymes and for their catalytic functions.

In an individually-variable population of micro-organisms, it is by no means easy to analyse the biochemical effect of metabolite analogues except in terms of a growth effect. In higher animals, the physiological and histological effects induced by vitamin (essential metabolite) deficiencies have been more fully characterised, and the effect of feeding a growth-inhibitory metabolite analogue has been analysed, not only in terms of growth inhibition, but also in terms of the pathological changes induced.

When pyrithiamin is fed to mice, symptoms characteristic of thiamin (vitamin B_1) deficiency are displayed and the condition can be cured by the feeding of a sufficient excess of thiamin (Woolley and White, 1943*a*). *Iso*riboflavin, the 5': 6-dimethyl analogue of riboflavin, when fed to rats, prevents growth and gives rise to symptoms characteristic of riboflavin deficiency (Emerson and Tishler, 1944); the phenazine analogue of riboflavin produces similar vitamin deficiency symptoms in mice (Woolley, 1944*b*); both these effects are reversed by feeding riboflavin. Mice do not normally require nicotinic acid in their diet, but feeding of 3-acetyl pyridine produced the symptoms known to occur in other animals fed on a nicotinic acid free diet (Woolley, 1945); the symptoms disappeared rapidly when nicotinic acid was added to the diet. A condition resembling scurvy can be induced by feeding glucoascorbic acid and the condition can be cleared up by addition of excess ascorbic acid to the diet

(Woolley, 1944*d*; Woolley and Krampitz, 1943). Symptoms of vitamin K deficiency, namely a large increase in the coagulation time of the blood, may be induced in the rat, an animal not normally affected by lack of this vitamin, by feeding dicumarol (Overman, Field, Baumann and Link, 1942). The condition does not develop when large quantities of vitamin K are fed along with dicumarol. The pantothenic acid analogues tested against malarial infections in chicks (p. 217) caused symptoms of pantothenic acid deficiency which were relieved by feeding pantothenate (Brackett, Waletzky and Baker, 1946).

With these few examples before us it is convenient to pause and take stock of the position. As already emphasised, competitive inhibition in isolated enzyme systems has been found to occur when a compound structurally similar to a natural substrate competes with that substrate for an active group on an enzyme surface. The characteristics of competitive inhibiton in enzyme systems were defined as follows :—

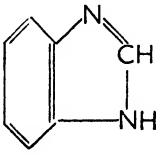
(1) At a fixed concentration of inhibitor the degree of inhibition is inversely proportional to substrate concentration ;
(2) for a given degree of inhibition the ratio of substrate concentration to inhibitor concentration is constant over a wide concentration range ;
(3) with a series of structurally related inhibitors acting on the same enzyme, inhibitions are proportional to the dissociation constants of the enzyme-inhibitor complexes.

It has been possible to apply these criteria to growth inhibition by metabolite analogues, and to reversal of growth inhibition by the related essential metabolites. The metabolite-inhibitor relationship is usually consistent with the assumption that the two compounds are in competition for a common site on some cellular enzyme. In other words, the metabolite analogue appears to prevent or reduce cellular utilisation of essential metabolite (*cf.* McIlwain and Hughes, 1945 ; McIlwain, 1945 ; Sarett, 1946). Where non-competitive antagonism exists, the

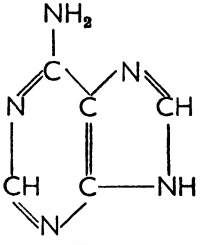
analogue may inhibit synthesis of the metabolite, as in the case of folic acid or tryptophan.

In the majority of cases so far examined, a metabolite analogue is only effective as a growth inhibitor for those strains of micro-organism which require an external source of the related essential metabolite ; thus, pyrithiamin inhibits at low concentrations only those organisms which require an external source of the intact thiamin molecule. It is less effective against organisms which can synthesise one half of the molecule, and practically ineffective against organisms which require neither the pyrimidine nor the thiazole " half." In a similar fashion, pantoyl-taurine only inhibits growth of organisms which normally require added pantothenic acid for growth ; pyridine-3-sulphonic acid only inhibits growth of strains which are unable to synthesise nicotinic acid. Desthiobiotin acts as a growth inhibitor for *Lactobacillus casei*, an organism which requires preformed biotin. In other less-exacting organisms, the same compound acts as a growth factor and a precursor of biotin (Lilly and Leonian, 1944 ; Dittmer, Melville and du Vigneaud, 1944) (see p. 141).

In contrast to the above group, the sulphonamides inhibit organisms which normally synthesise their own requirements of P.A.B.A., as well as those which require P.A.B.A. as a constituent of the medium. Sulphonamides inhibit conversion of both endogenous and exogenous P.A.B.A. into pteroylglutamic acid and so prevent growth of organisms which



Benzimidazole



Adenine

synthesise their own supplies of pteroylglutamic acid. Benzimidazole inhibits growth of organisms which do not require added adenine (Woolley, 1944*a*), and phenylpantothenone is

growth-inhibitory for organisms able to synthesise pantothenate. In the case of sulphonamides and benzimidazole, excess of the natural metabolite added to the medium reverses the growth-inhibitory effect of the analogue in a competitive manner. The question naturally arises—Why do sulphanilamide and benzimidazole inhibit growth irrespective of the growth requirements of the strain, although they are susceptible to antagonism by excess of the related essential metabolite ? Other metabolite analogues only inhibit organisms for which the metabolite is a growth factor.

It is impossible, as yet, to give a clear-cut answer to this question. If we visualise the possible explanations we may perhaps help to suggest an experimental approach which can provide an answer to the problem, and incidentally provide a guide to the synthesis of chemotherapeutically-effective metabolite analogues other than the sulphonamides.

A number of cases have been reported where biochemical mutation induced by exposure of a wild strain of the mould *Neurospora* to X-rays results in failure to synthesise a metabolite which is readily synthesised by the wild strain (see p. 108). The synthetic deficiency is often accompanied by a specific sensitivity to inhibition by metabolite analogues, which is lacking in other mutant strains and in the parent strain. The most striking case is the specific inhibition of a " lysine-less " mutant by arginine ; arginine does not inhibit wild-type *Neurospora*, and therefore does not block utilisation of endogenous lysine (Doermann, 1944). The different effect in the two cases can best be explained by the legitimate assumption that, in the wild type, arginine passes from the medium through the cell wall to reach the cellular metabolic system and is immediately metabolised ; it never reaches the site of utilisation of endogenously-synthesised lysine. In the mutant " lysine-less " strain, both arginine and lysine pass the cell wall together, and both have to be acted on by specific enzymes in the presence of one another. The close structural similarity between the two amino acids enables either to displace the other from its specific enzyme in a competitive manner, so that excess of arginine results in a failure of the cellular

metabolic system to take up sufficient lysine for protein synthesis.

This view of the course of events is supported by the results of McIlwain (1945) on the inhibition of pantothenate utilisation by pantoyl-taurine, and by those of Sarett (1946) on the inhibition of riboflavin utilisation by riboflavin analogues. In both cases, the metabolite analogue appeared to block utilisation of the metabolite by passing through the cell wall with the metabolite and arriving at the same time at the initial reaction site which is responsible for the uptake of the metabolite.

Presumably, metabolite analogues may fail to inhibit growth of organisms which synthesise their own supply of metabolite because, as in the case of arginine, the analogues are caught up in the metabolic system designed for the natural metabolite as soon as they pass the cell wall, and then are rapidly metabolised to a form which cannot interfere with the secondary utilisation of endogenously-synthesised metabolite. If this assumption is correct, there should be traceable a difference between the metabolic fate of anti-metabolites which are only effective growth inhibitors for organisms which require preformed metabolites (*e.g.* pyrithiamin), and anti-metabolites which inhibit growth of non-exacting as well as related exacting strains. In other words, an effective growth inhibitor would have to possess considerable biological stability such that it could not be readily converted by metabolic enzymes to an inactive form; presumably sulphanilamide, phenylpantothenone and benzimidazole fall into this category.

An artificially produced pyrithiamin-fast strain of yeast was found to have developed a metabolic system for the cleavage of pyrithiamin into its component parts; the system was also found in those organisms which normally synthesised their own requirements of thiamin (Woolley, 1944*c*). It seems probable therefore, that in the thiamin synthesisers the inhibitory analogue is metabolised to a harmless form, long before it reaches the vital site in the cell at which thiamin is synthesised and utilised intracellularly. The failure of pantoyl-taurine to inhibit organisms not requiring

preformed pantothenic acid may be due to its rapid hydrolysis within the cell, since indirect evidence has been obtained for the occurrence of a small amount of hydrolysis in the medium in contact with bacteria (Stansly and Alverson, 1946). This might also account for the fact that pantoyl-taurine was found to be a growth factor for a pantoic acid exacting strain of *Clostridium septicum* (Ryan, Schneider and Ballentine, 1947).

Another explanation of the failure of anti-metabolites to inhibit growth of organisms which synthesise their own requirements of related metabolite must also be considered. As we have seen, the growth-inhibitory action of a metabolite analogue is effectively antagonised by a sufficient concentration of the metabolite. Obviously, if a cell synthesises its own supply of metabolite, the concentration of that metabolite within the cell wall may be such that any metabolite analogue passing into the cell is effectively antagonised. Examples have already been quoted of the association of increased P.A.B.A. synthesis with increased resistance to sulphonamides. As will be indicated in the discussion of drug resistance (Chapter VI), all cases of increased resistance cannot be explained in terms of increased production of antagonist, so that each drug and each species of micro-organism must be regarded as a separate problem. One case has been examined in some detail (Woolley and White, 1943*b*). The amount of thiamin synthesised by those organisms for which it is not a growth factor was measured quantitatively; although apparently quite adequate for growth in absence of pyrithiamin, it was obviously insufficient to antagonise the quantities of pyrithiamin used in the inhibition tests.

In this brief outline of competitive metabolite antagonists it has not been possible to detail the numerous examples scattered throughout the chemical, biochemical and medical literature, or to deal with the recent successful clinical development of histamine antagonists. More detailed accounts of the whole subject by Roblin (1946) and Woolley (1947) are available.

Possibilities and limitations of metabolite analogues

As we have pointed out already, many metabolite analogues cannot be used chemotherapeutically because they produce specific vitamin deficiency diseases in the host animal. Such a difficulty is inevitable in the design of new chemotherapeutic drugs, since these are often structural analogues of metabolites essential to host as well as to parasite. However, the success achieved in rendering less toxic the arsenoxides and pentavalent organic arsenicals and antimonials, should encourage development of new metabolite analogues, even when first attempts have led to drugs which are equally toxic to parasite and host. The selective parasiticidal action of organo-metallic drugs must depend very largely upon selective distribution, which favours concentration of the drug in parasite rather than host cells. Some success has been achieved in lowering toxicity, for the host, of pantothenate analogues, without impairment of their antimalarial activity (Brackett, Waletzky and Baker, 1946).

The ideal approach to chemotherapy would be through obstruction of an enzyme system or metabolite essential to parasite but non-existent in or less essential to host. Among the sulphonamides we have stumbled accidently against one such group. Following upon the recognition of folic acid (pteroylglutamic acid) as a derivative of P.A.B.A., and the demonstration that sulphonamides prevent synthesis of pteroylglutamic acid, it is apparent that only cells which are obliged to synthesise pteroylglutamic acid will be highly susceptible to sulphonamides. Cells which draw preformed pteroylglutamic acid from their medium will be more resistant. Various Gram-negative bacteria and animal cells belong to the latter category. If dosage of sulphonamide is too high or too prolonged, those cells of the host which require to multiply rapidly (the cells of the bone marrow), may show signs of inhibition of cell division, possibly because their supply of folic acid is slowly exhausted. Granulocytopenia induced by sulphonamides is rapidly cured by administration of folic acid (Daft and Sebrell, 1945).

In seeking reactions specific to parasite and absent from host, that group of enzymes and coenzymes concerned primarily with energy-yielding oxidative reactions (about which we know most) does not look promising, since micro-organisms and higher animals metabolise the same foodstuffs by closely similar pathways. Proteins are, however, species-specific and therefore some species-specific synthetic processes must go on during growth to build up their patterns. A logical point of attack would then seem to be the mechanisms concerned with protein synthesis. Surprisingly little evidence is available to show whether the recognised amino acids are all common to animal and bacterium, and a systematic investigation of protein from micro-organisms seems highly desirable. To upset protein synthesis, the organism might be presented with amino acid analogues capable of being built up in peptide linkage but incapable of proper function in the completed protein. Alternatively, peptides might be synthesised capable of inhibiting by their unnatural configuration the build-up or breakdown essential for vital function. The recognition of gramicidin as a peptide and of penicillin and streptomycin as closely related to amino acids is suggestive of further profitable exploration in this field (Work, 1948).

Provided we know enough about the building blocks used by micro-organisms, we may be able to imitate these sufficiently closely for the analogue to be caught up in a synthetic process for which it is unsuited. Before a metabolite analogue can play such a part, it must, however, be sufficiently stable to resist breakdown during metabolism and it must also resemble a natural metabolite so closely that an enzyme, a highly selective and specific catalyst, must be unable to reject it in preference to its natural substrate.

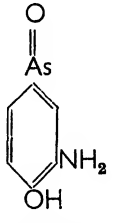
The chemotherapy of virus infections is still very much in the embryonic phase. Little is known of the biochemical activities of viruses. A possible line of chemotherapeutic attack has been suggested by a study of the nature of combination between virus and host. In the case of certain bacterial viruses which infect *Escherichia coli*, tryptophan is a necessary co-factor for adsorption of virus on the host cell

(Anderson, 1945). 5-Methyltryptophan appeared to have an action somewhat similar to tryptophan in that it permitted adsorption of virus on host, but in the presence of this tryptophan analogue the infected cell was not a suitable milieu for virus multiplication (Cohen and Anderson, 1946).

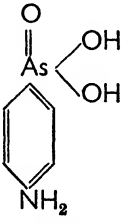
Available evidence suggests that the influenza virus, when it causes hæmagglutination, may react with a carbohydrate group in red cells. Green and Woolley (1947) tested other carbohydrates for their ability to inhibit the reaction by competition with the red cell carbohydrate for carbohydrate-receptor groups on the virus. A number of polysaccharides, particularly apple pectin, were found to be capable of inhibiting hæmagglutination; apple pectin also inhibited growth of the virus in chicken embryo. As the authors themselves point out—"Although the working hypothesis just outlined has led directly to positive experimental results, it does not necessarily mean that this hypothesis is the correct one."

Antagonism and the mode of action of related drugs

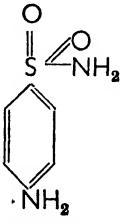
The study of competitive inhibition has provided a useful indication whether two related drugs which are toxic to the same organism are acting at the same key point. Peters (1943) found that both atoxyl and sulphanilamide inhibited growth of *Escherichia coli* on a synthetic medium. In both cases the effect was fully reversed by P.A.B.A. However, sulphanilamide had no trypanocidal effect, while the trypanocidal effect of atoxyl was reversed, not by P.A.B.A., but by cysteine. It is



Mapharsen



Atoxyl



Sulphanilamide

evident that while atoxyl and sulphanilamide act in the same way on *E. coli*, the toxic effect of atoxyl on trypanosomes is due

to a quite different mechanism. Mapharsen, an effective trypanocide, is also toxic to *E. coli*, but in this case the effect is not reversed by P.A.B.A.; thus, we apparently have two possible modes of action of organic arsenicals on *E. coli*. Knowing these facts, we can conclude that modifications in structure which lead to enhanced trypanocidal activity among arsenoxides are unlikely to be profitable in increasing bacteriostasis of *E. coli* by sulphonamides or even by atoxyl. Trypanocidal arsenoxides in general are antagonised in their trypanocidal action by glutathione or cysteine, but not by P.A.B.A. An exception to this was found in γ-(*p*-arsenophenyl)-butyric acid which was antagonised by both compounds (Williamson and Lourie, 1946). The only explanation for this action is to assume that the drug acts ultimately in the same manner as other trypanocidal arsenicals, but that P.A.B.A. may be preventing or limiting admission into the trypanosome cell (see also p. 296).

Similar studies have shown that the antimalarial activity of certain sulphonamides is not related to their similarity to P.A.B.A. The antimalarial activity of 2-metanilamido-5-chloropyrimidine and related metanilamides was not antagonised by P.A.B.A. The sulphadiazine analogue with a 5-halogen in the pyrimidine ring (2-sulphanilamido-5-bromopyrimidine) was bacteriostatic for *Escherichia coli* and this action was completely antagonised by P.A.B.A. The same compound was also active against malaria (*Plasmodium gallinaceum*), but this action was only partially antagonised by P.A.B.A. (Brackett and Waletzky, 1946; English, Clark, Clapp, Seeger and Ebel, 1946).

Non-specific competitive antagonism

When two related dyes, one of which is an enzyme inhibitor, are added simultaneously to an enzyme preparation, the degree of inhibition observed may be less than when the inhibitory dye is added alone. The harmless dye is in this case competing with the inhibitory dye for the same point on the enzyme surface and so reducing competitively the degree of inhibition. Numerous examples of this effect are

recorded in the literature of enzymology. The only difference between this type of competition and that which we have already discussed lies in the nature of the antagonist. In specific competitive inhibition, the antagonist is a natural substrate (metabolite) or coenzyme; in non-specific competitive inhibition, neither inhibitor nor antagonist is related chemically to the substrate or coenzyme.

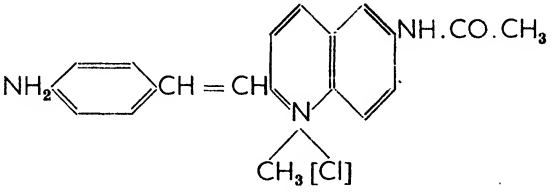
The effect of *p*H on the degree of inhibition of enzymes by basic dyes can be attributed to competition between the hydrogen ions and basic dye ions for certain acidic groups on the enzyme surface. Hydroxyl ions may similarly compete with, and reduce inhibition caused by, toxic anions; such cases are discussed more fully in Chapter VII. Two basic or two acidic drugs may also compete with one another.

There are numerous instances of analogous effects in the field of chemotherapy. Browning and Gulbransen (1922) reported that the trypanocidal action of trypaflavine was reduced by parafuchsin when mice, infected with a parafuchsin-resistant strain of trypanosome, were fed with parafuchsin and the infection treated with trypaflavine. Browning suggested the term "*therapeutic interference*" for this type of effect. Similar results were obtained with normal trypanosomes; the action of a variety of trypanocidal drugs, both arsenicals and acridines, was antagonised by parafuchsin and other dyes (Schnitzer, 1926; Schnitzer and Rosenberg, 1926 *a* and *b*; Schnitzer and Silberstein, 1926 *a* and *b*). Hasskó (1935) showed that the protective dye lessened the degree of absorption of acriflavine by trypanosomes.

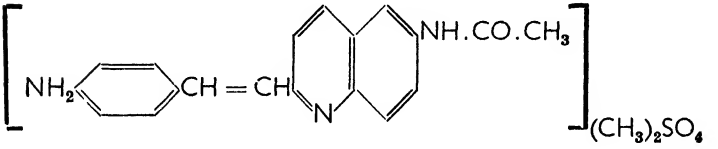
Interference phenomena can also be demonstrated *in vitro*. Carbon dioxide production from glucose by yeast was inhibited by acriflavine or methyl violet. Methyl violet alone, in sufficiently low concentrations, had no effect, but it caused considerable reduction of the inhibitory effect of acriflavine. Preliminary staining with low concentrations of acriflavine could similarly reduce the toxic effect of high concentrations of methyl violet (Wright and Hirschfelder, 1930).

An interesting case of non-specific competitive inhibition arose from the synthesis of trypanocidal styrylquinolines

(Browning, Cohen, Ellingworth and Gulbransen, 1926, 1929; Browning, Cohen, Cooper and Gulbransen, 1932). An attempt to prepare 2-(*p*-aminostyryl)-6-acetylaminoquinoline methochloride by two different methods resulted in two related compounds, one a quaternary salt (I), the other an addition compound of methyl sulphate and base (II). Compound I



I



II

was an effective trypanocide in mice infected with *Trypanosoma brucei*; compound II had no trypanocidal action, but was found to antagonise the action of compound I when present in one-tenth the concentration of I. Evidently compound II, although inactive therapeutically, resembled I sufficiently closely to be absorbed in the same way.

Probably the same type of effect is involved in the reversal of atebrin, quinine and propamidine bacteriostases by spermine, spermidine and other polyamines (Snell, 1944*a*; Silverman and Evans, 1944). The difficulty involved in the interpretation of observed results is well illustrated by this case. *Pseudomonas pyocyanea* was known to oxidise polyamines, including spermidine, and it was reasonable to suppose that inhibition of growth by bacteriostatic bases was due to inhibition of metabolism of the essential growth factor, spermidine. Such a mechanism would necessitate classifying the effect as specific competitive inhibition. Other facts, however, indicate that a non-specific mechanism is involved. In *Escherichia coli*, spermidine was not oxidised or required as a growth factor,

but successfully antagonised the bacteriostatic action of propamidine. Certain soil bacilli, capable of growing in a salt-glucose medium containing ammonium phosphate as sole source of nitrogen, were inhibited by high concentrations of atebrin and the bacteriostasis was antagonised by spermidine. Other related organisms incapable of growing in glucose-salt medium were inhibited by low concentration of atebrin, but the bacteriostasis was not antagonised by spermidine. In Chapter IV (p. 165) we showed that atebrin and quinine inhibit a large number of enzymes, probably because of their basic nature; polyamines might well antagonise them by displacement.

Lecithin, also, is capable of antagonising the antibacterial action of diamidines (Elson, 1944) and has a similar protective effect against cationic and anionic detergents (Baker, Harrison and Miller, 1941*b*) (see Chapter VII).

It is well known that the bactericidal action of dyes depends greatly upon the *p*H of culture media (Browning, Gulbransen and Kennaway, 1919). Stearn and Stearn (1924, 1926) suggested that dyes exercised their bacteriostatic effect mainly in the ionised condition; thus, the cation of a basic dye was visualised as acting by being adsorbed on an acidic group of a cellular protein. Such a mechanism allows *p*H effects to be explained on the basis of non-specific competitive inhibition. On increase of H ion concentration, some of the dye cation will be displaced from its combination with protein by the harmless H ion, so permitting cellular enzymes to resume their normal functioning. In confirmation of their interpretation, Stearn and Stearn pointed out that, among a series of related dyes, increasing bacteriostatic action was associated with increasing base strength. Much evidence not available to the Stearns in 1925 has since tended to strengthen the belief in their interpretation (see Chapter VII). McCalla (1940) showed by a series of quantitative experiments, that an actual rapid exchange of all types of cations could take place between the bacterial cell and surrounding medium. Non-specific competitive antagonism may then be regarded as an ion-exchange phenomenon in many cases.

The effect of *p*H change on the activity of widely differing types of acidic and basic drugs is in agreement with the ion exchange interpretation. Hydrogen ions antagonise competitively acridine, propamidine, atebrin, streptothricin and cationic detergents. Elson (1945) gives a diagram, typical of the type of effect observed, for the effect of *p*H change on the antibacterial activity of propamidine (Fig. 24). (For full discussion of effect of *p*H on drug action see Chapter VII).

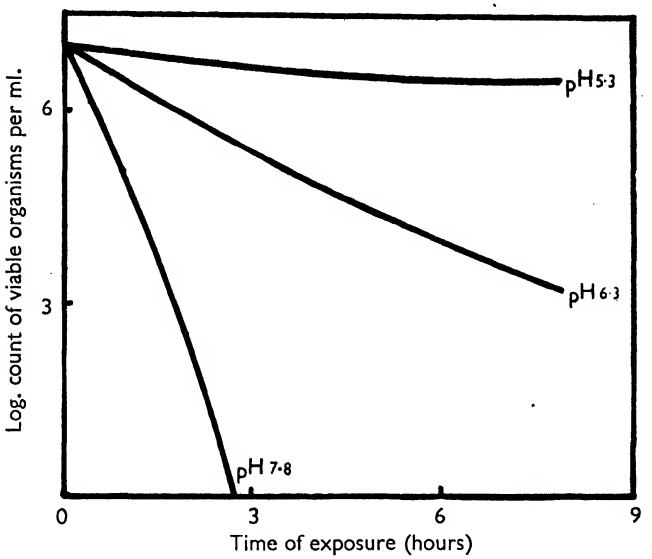


FIG. 24.—Effect of *p*H on the bactericidal action of propamidine. (Elson, 1945.)

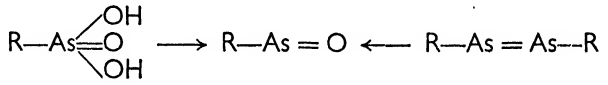
It is apparent that no clear-cut line can be drawn between specific and non-specific competitive inhibition, but it is very necessary, when elaborating chemotherapeutic theories based upon nutritional requirements of micro-organisms, to remember that every drug antagonist is not automatically an essential metabolite.

Antagonism of drugs containing arsenic, mercury and antimony

The organic derivatives of arsenic and antimony, used extensively in the treatment of protozoal and spirochætal infections, are also toxic to bacteria and, indeed, to most

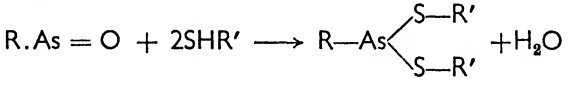
forms of life, including the animal host. Probably the toxic action resides in the metallic portion of the molecule, while the organic " tail " influences distribution and specificity.

Much of the credit for the development of a satisfactory theory of the mode of action of these drugs is due to Voegtlin, but the possibility that arsenicals might be toxic because of their affinity for mercapto groups had been suggested earlier by Ehrlich (1909). Voegtlin concluded that organic arsenicals were immediately toxic only in the arsenoxide form. During the considerable latent period before development of trypanocidal activity, atoxyl and arsphenamine were slowly converted by the tissues of the host to the arsenoxide form (Voegtlin, Dyer and Leonard, 1923).

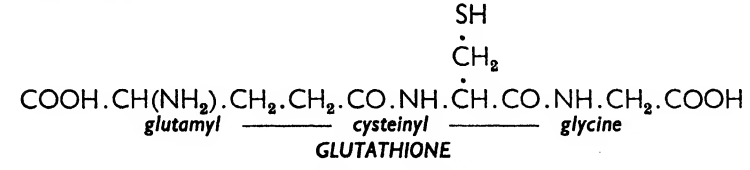


This view has recently been supported by careful analytical work on the excretion of pentavalent and trivalent phenyl arsenoxides after injection into rabbits. Although the amount of trivalent phenyl arsenoxide excreted after injection of the pentavalent compound was small, it was sufficient to account for the toxicity of the latter on the theory that it must be reduced in the body before producing its lethal effect (Crawford and Levvy, 1947).

Voegtlin was able to show that arsenoxides reacted readily with hydrogen sulphide and mercaptoacetic acid as follows :—



At the time when this study on the mode of action of arsenical drugs began, Hopkins had just discovered glutathione and



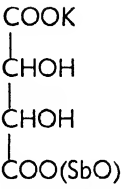
had shown that it was widely distributed in living tissues. This ubiquity, combined with the existence in glutathione of

a highly reactive sulphydryl group, suggested to Voegtlin that arsenicals might be acting by combination with glutathione or other similar sulphydryl compounds essential to the life of the cell. He was able to show that trypanosomes contained free sulphydryl groups and that sodium thioglycollate ($SH.CH_2COONa$), cysteine ($SH.CH_2.CH(NH_2).COOH$) and glutathione antagonised the arsenoxide inhibition of motility of trypanosomes *in vitro*. *In vivo*, the injection of sulphydryl compounds one minute before injection of arsenoxide was found to slow considerably the rate of destruction of trypanosomes; the effect was not permanent, but the decline in arsenoxide-antagonistic action was found to coincide with a decline in the concentration of injected thiol compound in the blood. The oxidised forms of the sulphydryl compounds such as cystine or dithioglycollate showed comparatively little arsenoxide-antagonistic action. Sulphydryl compounds were found to lower the toxicity of arsenoxides for animals as well as for trypanosomes; thus, after administration of a lethal dose of arsenoxide, the life of an animal could be prolonged for several days by injection of antagonist. Voegtlin concluded that arsenic in the trivalent form *is a specific poison affecting the SH groups of the protoplasm.*

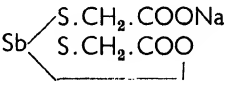
This view has been amply confirmed and extended during the intervening years. The bactericidal action of mercuric chloride had long been known to be reversed by treating the poisoned organisms with hydrogen sulphide or ammonium sulphide (Geppert, 1889; Chick, 1908). Fildes (1940*a*) showed that sulphydryl compounds such as glutathione and cysteine also antagonised the bactericidal effect of mercuric chloride. Mapharsen has been shown to possess antibacterial action comparable to that of mercuric chloride (Albert, Falk and Rubbo, 1944), and its bacteriostatic effect is also antagonised by sulphydryl compounds. Pentavalent arsenicals do not possess similar bacteriostatic properties.

Recently it has been found that both the trypanocidal action and the systemic toxicity for animals of trivalent antimonials such as tartar emetic and antimony thioglycollate are antagonised by cysteine. The toxicity of pentavalent

antimonials is not affected (Chen, Geiling and MacHatton, 1945).



Tartar emetic



Sodium antimony thioglycollate

It is firmly established, then, that the toxic effect of metal complexes upon micro-organisms is antagonised by naturally-occurring sulphydryl compounds, and it is tempting to suggest with Fildes (1940*a*) that the organometallic compounds are toxic because they deprive cells of their essential sulphydryl-containing metabolites. Another, and we believe more probable explanation, can be profitably considered. As shown by Cohen, King and Strangeways (1931), the thioarsenites are largely hydrolysed in weak solution, and it seems improbable, if this is so, that the low concentration of trivalent arsenical reaching a pathogen *in vivo* would deprive it of any large percentage of the available glutathione or cysteine.

Arsenoxides inhibit the respiration of kidney, liver, testis, rat sarcoma and yeast, as well as trypanosomes and other pathogenic organisms; the inhibition is reversed in each case by glutathione (Voegtlin, Rosenthal and Johnson, 1931). Arsenoxides and glutathione have the same effect on the motility and viability of trypanosomes as they have on respiration. Trivalent organic arsenicals are bound by proteins which give a positive nitroprusside test, but do not combine with proteins which give no nitroprusside test (Rosenthal, 1932). We have already discussed in Chapter IV the importance of sulphydryl groups for enzymic activity. Some enzymes, known as "SH enzymes," have been shown to possess in the undenatured state several exposed sulphydryl groups which are absolutely essential for enzyme activity; such enzymes give a positive nitroprusside test. About thirty "SH enzymes" are now recognised, among them several concerned with carbohydrate metabolism (Barron and Singer, 1945

a and *b*). This suggests that arsenoxides may act directly by combination with enzyme proteins rather than by depriving the cell enzymes of essential metabolites.

If arsenoxides act *in vivo* by combining with the essential groups of " SH enzymes," it may well be asked why they are capable of destroying trypanosomes and other pathogens in the living animal, without at the same time inhibiting the essential " SH enzymes " of the host. Some enzymes, although possessing sulphydryl groups, are completely unaffected by concentrations of arsenoxide which inhibit such sensitive enzymes as yeast alcohol dehydrogenase, urease, hexokinase and phosphoglyceraldehyde dehydrogenase. The selective destruction of trypanosomes may be also attributed to selective distribution of trypanocidal drugs, which are, however, not entirely without toxic action for the host. Eagle and Magnuson (1944) have found *in vitro* a general correlation between the trypanocidal activities of a wide range of phenylarsenoxides and the amounts of drug taken up by trypanosomes (Table 14). Selective distribution *in vivo* is, however, only an end-result of a number of variable factors (see Chapter VII).

When two types of cells metabolise substrates by the same pathway it does not follow that the enzymes catalysing intermediate reactions are necessarily identical. Alcohol oxidase of liver catalyses the same reaction as alcohol oxidase of yeast, but the former enzyme is not inhibited by iodoacetamide or arsenoxide while the latter is highly sensitive, *i.e.* is an " SH enzyme." The particularly high rate of carbohydrate metabolism of some trypanosomes is suggestive. Several of the reactions of glycolysis, pyruvate oxidation and carbon dioxide fixation are catalysed by " SH enzymes," and readily inhibited by arsenoxides. Only those species of trypanosome which have a high rate of carbohydrate metabolism can be successfully destroyed *in vivo* with arsenical drugs. The selective action of trypanocidal arsenicals may therefore be partly attributable to the relatively greater importance of " SH enzymes " in parasite than in host.

In addition to arsenoxides and iodoacetamide, numerous other organic molecules may react readily with the active

hydrogen of "SH enzymes." Quinones possess antibiotic properties which have been attributed to their ability to react

TABLE 14

The trypanocidal activity of arsenicals in relation to their binding by trypanosomes

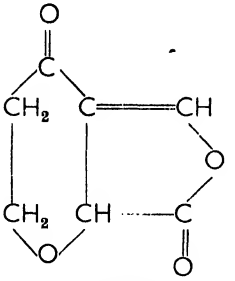
(Final arsenic concentration of medium was 1·66 μg./c.c.)

Compound. (All compounds are phenylarsenoxides $R\text{-}C_6H_4AsO$ unless otherwise stated.) R =	Relative trypanocidal activity *in vitro* (molar) referred to phenylarsenoxide as 100.	Average concentration in trypanosomes (mgs. As per cent.)
p-SO_3H	0·06	0·41
p-$CONHCH_2COOH$	0·22	0·36
p-$SO_2NHCH_2CONH_2$	1·4	0·68
p-$CH_2CONHCH_2CONH_2$	1·5	1·4
p-$CH{=}CHCOOH$	2·0	0·2
3-$NHCOCH_3$-4-OH	3·0	2·1
p-OCH_2COOH	4·5	1·5
p-CH_2COOH	4·7	1·6
p-$CONHCH_2CONH_2$	15·0	5·7
p-OCH_2CONH_2	26·0	6·8
3-NH_2-4-OH	27·0	6·5
3-OH-4-NH_2	30·0	6·9
p-$NHCOCH_2NH_2$	31·0	3·6
p-$SO_2N(C_2H_5)_2$	35·0	7·4
p-$NHCONH_2$	35·0	5·3
p-SO_2NH_2	30·0	5·5
p-$CONH_2$	45·0	7·7
3-NH_2-4-$CONH_2$	52·0	7·6
p-$(CH_2)_3COOH$	54·0	6·9
3-NH_2-4-Cl	59·0	8·7
m-OH	66·0	8·2
2-OH-5-AsO-azobenzene	71·0	9·6
1-naphthylarsenoxide	79·0	10·4
2, 4-diCl	80·0	9·5
p-Cl	90·0	7·2
o-CH_3	91·0	9·5
o-Cl	92·0	9·3
m-Cl	95·0	10·3
phenylarsenoxide	100·0	9·7
p-CH_3	102·0	10·0
2-naphthylarsenoxide	105·0	10·2

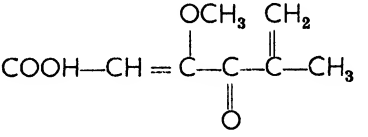
Fom Eagle and Magnuson (1944).

with sulphydryl groups (Colwell and McCall, 1945). This interpretation, although supported by the reversal of certain cases of quinone bacteriostasis by thiols such as cysteine or thioglycollate, cannot be considered as entirely satisfactory.

Geiger (1946) found that, although in general, quinones were less active against Gram-negative bacteria than Gram-positive, reversal by thiols was only effected in the case of Gram-negative organisms. He concluded that the activity against Gram-negative organisms was associated with an unsubstituted position ortho to the carbonyl groups, presumably necessary for combination with sulphydryl groups. No specific structure in the quinone molecule was necessary for activity against Gram-positive organisms, where it appeared that sulphydryl groups were not specifically attacked. In the case of hydroquinones, antagonism by thiols was also noted only with Gram-negative organisms. The reaction of thiols with quinones is an example of a general reaction, the addition of thiol to $\alpha : \beta$-unsaturated ketone. Other substances, both natural and synthetic, containing the $\alpha : \beta$-unsaturated ketone grouping have been found to possess antibacterial action which was antagonised by cysteine. The antibiotics clavicin and penicillic acid were found to abolish the nitroprusside reaction of



Clavicin



Penicillic acid

cysteine, showing that they reacted with the sulphydryl group, and their antibacterial actions were fully antagonised by thiols. The synthetic ketone acrylophenone showed similar antibacterial action, also antagonised by thiols (Geiger and Cohn, 1945; Cavallito, Bailey, Haskell, McCormick and Warner, 1945; Rinderknecht, Ward, Bergel and Morrison, 1947).

In Chapter IV we discussed the reversibility of enzyme inhibition, and showed that the toxic action of an inhibitor was dependent on the dissociability of the inhibitor-enzyme

complex relative to that of the antagonist-enzyme or antagonist-inhibitor complex. The effectiveness of 2 : 3-dimercaptopropanol (B.A.L.) in antagonising war gases, such as lewisite, which combine with " SH enzymes," was shown to be due to formation of highly stable non-dissociating ring compounds with the poisons. The stability of these compounds is such that B.A.L. can reverse the toxic and vesicant effect of lewisite for at least an hour after contamination of the skin, after which time irreversible changes presumably occur. B.A.L. can also counteract the toxic effect of arsenical drugs on animals (Stocken, Thompson and Whittaker, 1947).

The failure of B.A.L. to reverse the toxic reaction to lewisite if applied to the skin more than one hour after lewisite, is characteristic of many antagonists both *in vitro* and *in vivo*. We showed that enzymes may be reactivated after poisoning with mercury or arsenicals, provided the antidote is added soon enough after the poison. If bacteria or other unicellular organisms are brought into contact with dilute mercuric chloride, growth and reproduction are immediately inhibited, but provided cells are not left too long in contact with mercuric chloride, growth is resumed when mercury is removed by hydrogen sulphide or glutathione (Chick, 1908 ; Fildes, 1940*a*).

It will be noted that the thiol-antagonism of organometallic compounds and heavy metals does not involve competitive inhibition as defined at the beginning of this chapter. Competitive inhibition is regarded as occurring when two substances *which cannot interact chemically* compete for a common centre on an enzyme. Thiols antagonise heavy metals, not by competing with them, but by a chemical combination. The reversal of acridine bacteriostasis by nucleate (McIlwain, 1941*a*) and the antagonism of stilbamidine and pentamidine bacteriostasis by the same type of compound (Bichowsky, 1944) may be, as suggested by McIlwain, due to the ability of nucleate to function as an essential metabolite. The effect can be equally well explained on the basis of chemical combination of basic drug with

acidic nucleic acid (Massart *et al.*, 1947). The large anion of sodium dodecyl sulphate can antagonise the bacteriostatic action of atebrin (Valko and DuBois, 1944), but in this case, there can be no question of the dodecylsulphate ion acting nutritionally..

Quantitative aspect of combination of metals with cells and enzymes

Heavy metals produce lethal effects on living cells at extremely low concentrations. When first discovered, this general lethal action was considered so remarkable that it was given the special name "oligodynamic action" and many elaborate theories were advanced to explain its nature (for references see Buchanan and Fulmer, 1928). The low concentrations are deceptive, however, since living cells suspended in dilute solutions of metallic salts fix quite large quantities of metal before lethal action is produced. Quantitative measurement of the fixation of mercuric chloride by yeast (Herzog and Betzel, 1911) showed that there was a considerable concentration of mercury in the cell at the time of death. Spirogyra were killed by silver at a concentration of 3γ per litre, but the killed organisms were found to have fixed up to 60γ silver per gm. dry weight (Freundlich and Söllner, 1928). Quantitative measurement of the fixation of mercuric chloride by staphylococci also indicated that at the time of death there was a considerable concentration of metal in the cell (Liese and Mendel, 1923). A direct stoichiometric relation was found between the number of titratable thiol groups of *E. typhosa* and the amount of mercurial antiseptic required to sterilise cell suspensions. If sufficient time of contact were allowed, only one quarter of the bacterial thiol groups had to be blocked to effect complete sterilisation (Loureiro and Lito, 1946). The action of metals on cells is a double effect, firstly rapid fixation which is reversible, and secondly slow irreversible injury which can probably be attributed to general protein denaturation. The initial reversible fixation resembles very closely the action of heavy metals on "SH enzymes." Thus, Sumner and Myrbäck (1930) found that

purified urease was inactivated reversibly by 1 gm. atom of silver per 40,000 g. of enzyme, but a slow secondary irreversible combination with a further 10 gm. atoms of silver could take place. As noted already, denaturation of urease and other enzymes unmasks additional sûlphydryl groups, so that the course of the reaction can be regarded as rapid combination of metal with exposed essential sulphydryl groups, followed by slow denaturation of enzyme with accompanying increase in power to bind metal. A similar effect can be observed with arsenic poisoning of trypanosomes. Living trypanosomes fix arsenic reversibly ; after death, cells fix about ten times as much arsenic (Reiner, Leonard and Chao, 1932). The initial reversible combination of arsenic with essential groups of " SH enzymes " prevents cell multiplication or metabolism ; this causes death of the cell, protein denaturation and unmasking of additional sulphydryl groups.

Antagonism by amino acids

Various cases have been reported in which the antibacterial effects of drugs other than sulphonamides are strongly antagonised by certain amino acids. These results are difficult to interpret in terms of cell metabolism. Amino acids are essential building blocks for proteins, and it may be that their presence in the medium eliminates the need for certain synthetic reactions which are blocked by growth-inhibitory drugs. Methionine antagonism of sulphonamides has already been discussed. Woolley (1946*a*) reported antagonism of phenylpantothenone by amino acids ; histidine was the most active, and glutamic acid next. Penicillin-insensitive Gram-negative bacilli were found by Shwartzman (1946) to be sensitive to the drug in salt-glucose medium. Penicillin antagonism, in this medium, was shown by dicarboxylic mono-amino acids, and by arginine, histidine, hydroxyproline and cystine. Resistance of organisms to penicillin could be changed by repeated culture in the presence of antagonistic amino acids.

Arginine was found by Gale (1945) to have an antagonistic effect on sulphanilamide for certain streptococci which are

relatively insensitive to the sulphonamides. This effect was traced to the fact that the organisms in question require carbon dioxide for growth ; this can be supplied from arginine by decarboxylation. When the arginine in the medium is exhausted, carbon dioxide is obtained from other sources. This utilisation of carbon dioxide is inhibited by sulphanilamide, but not by sulphapyridine or sulphathiazole, and, in the absence of carbon dioxide and arginine, the organism is relatively susceptible to sulphanilamide, but the sulphapyridine and sulphathiazole resistance are unchanged.

The study of antagonists has led also into the field of drug resistance. It has been found possible to develop organisms resistant to a drug by growth in a medium from which the drug antagonist is omitted ; in other words, resistance may be developed by training an organism to be independent of the essential metabolite of which it would be deprived by addition of drug. Thus, *Corynebacterium diphtheriæ* developed resistance to pantoyl taurine when trained to grow in pantothenic acid-deficient media (McIlwain, 1943*b*). The inhibitory effect of indole-3-acetic acid on growth of *Strep. fœcalis* (antagonised by tryptophan) was not apparent when the organism was trained to grow on ammonia instead of amino acids (Perlman, 1946).

CHAPTER VI

DRUG RESISTANCE

Variation

THE enzymic balance of the bacterial cell can undergo considerable variation in response to changes in environment. *Escherichia coli* produces up to 20 times more invertase when it is grown in a medium containing sucrose than when the medium contains glucose (Karström, 1938). As a result of successive subculture under suitable conditions, an organism may develop a capacity to metabolise substances which were previously toxic to it, or it may develop the ability to synthesise an essential metabolite which it formerly required as an addition to the basal medium (Fildes, Gladstone and Knight, 1933).

In general, two types of enzymic change are recognisable, a rapid development of enzyme activity without the necessity of cell division, and a much slower alteration in metabolic pattern which can only be demonstrated over a series of generations. Rapid variation in enzymic balance in response to the presence of a given substrate in the culture medium is usually equally rapidly reversible. This type of variation we shall define as *variation by enzyme adaptation.* Variations which can only be observed after a series of subcultures are of a more permanent nature. The change has been brought about by a slow process of selection of variants which differ enzymically from the parent cells in such a way that they are better suited to the new environment. Eventually they are present in preponderant amounts, having overgrown the other cells of the culture which are less suited to the environment. This type of variation we shall define as *variation by selection.* Enzymic variation produced by selection during growth of successive generations may be permanent even in the absence of the selective mechanism, or it may be lost slowly over a number of subcultures.

Variation by adaptation.—Enzyme adaptation was fully investigated by Karström (1938), who distinguished between "adaptive" enzymes which appeared in response to contact of the cell with a suitable substrate, and "constitutive" enzymes which were always present in the cells of a given strain irrespective of the composition of the medium. Karström was able to show that organisms grown for a few hours in the presence of sugars which they did not normally ferment, adapted themselves to ferment these sugars. An example of

TABLE 15

Adaptation of Betacoccus arabinosaceus *to ferment sugars*
(+ = fermentation; 0 = no fermentation)

Betacoccus grown in presence of	Sugars subsequently fermented					
	Glucose, Fructose, Mannose	Galactose	Arabinose	Sucrose	Maltose	Lactose
Glucose .	+	0	0	(+)	0	0
Galactose	+	+	0	+	0	0
Arabinose	+	0	+	+	0	0
Sucrose .	+	0	0	+	0	0
Maltose .	+	0	0	+	+	0
Lactose .	+	+	0	+	0	+
No carbohydrate	+	0	0	+	(+)	0

From Karström (1938).

the adaptation of *Betacoccus arabinosaceus* (*Leuconostoc mesenteroides*) to ferment various sugars is given in Table 15.

It seems unlikely that completely new enzymes are elaborated by the cell in a few hours in response to external stimuli, and the difference between constitutive and adaptive enzymes is probably quantitative rather than qualitative. Contact with a suitable substrate would not necessarily result in formation of a new enzyme, but could increase the rate of synthesis or efficiency of utilisation of an enzyme already present in small amounts, or decrease its rate of destruction.

The work of Mirick (1943) on an adaptive enzyme of certain soil bacilli illustrates the general characteristics of adaptive enzymes. The bacilli grown in the absence of *p*-aminobenzoic acid (P.A.B.A.) possessed a small but measurable capacity to oxidise P.A.B.A. (see Fig. 25). When cells were grown in media containing P.A.B.A. their capacity to oxidise the acid increased rapidly in proportion to the concentration of

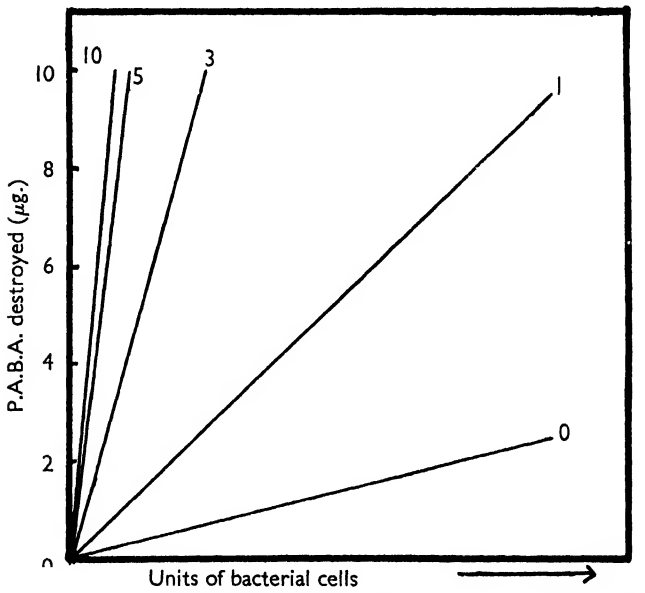


FIG. 25.—Activity of a soil bacillus in destruction of P.A.B.A. after twelve hours' growth in media containing varying amounts of P.A.B.A. as sole source of organic nitrogen. Figures represent μg. of P.A.B.A. per ml. of growth medium. (Mirick, 1943).

P.A.B.A. in the medium, but subculture in another medium containing no P.A.B.A. resulted in a rapid loss of activity. The acquisition of the power to oxidise P.A.B.A. was not dependent upon cell division, since shaking a washed suspension of non-adapted cells for one hour with P.A.B.A. in phosphate buffer resulted in a marked increase of enzymic activity.

The occurrence of enzyme adaptation without cell division has been demonstrated with other organisms and other enzymes, thus confirming that it is a process independent of

natural selection (Stephenson, 1939; Dubos, 1940; Gale, 1943; Spiegelman, Lindegren and Hedgecock, 1944).

The rate of adaptation depends not only on the nature of the culture medium on which the inoculum has been grown, but also upon the age of the cells. As is indicated in Fig. 26, *Strep. lactis* cells from a one-hour culture adapted themselves to ferment galactose in one hour, whereas cells taken from the same stock culture after four hours required nine hours to adapt themselves to ferment galactose. The one-hour cells

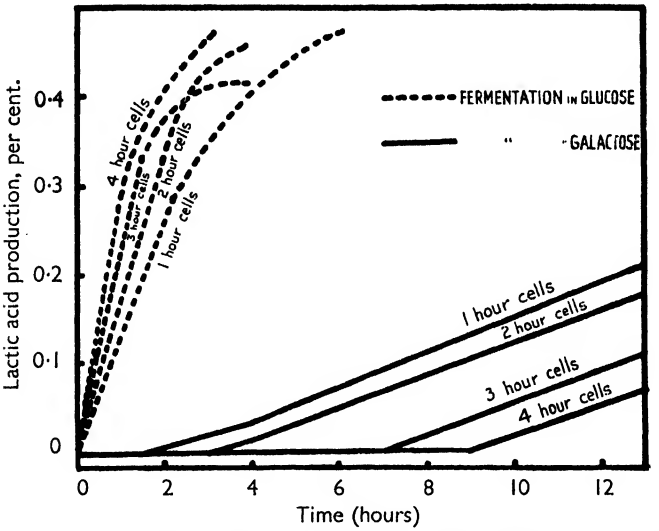


FIG. 26.—Adaptation to ferment galactose by *Strep. lactis* grown on glucose. (Hegarty, 1939).

were just coming out of the lag phase and the four-hour culture was past the middle of the logarithmic phase of growth (Hegarty, 1939).

Enzyme adaptation is a direct response to environment, and its function may be largely to counteract or utilise unfavourable external conditions. Thus, adaptive enzymes may act in such a way as to restore the *p*H of a medium to near neutrality, as is the case with the deaminases and decarboxylases of *Escherichia coli* (Gale and Epps, 1942); or they may act as detoxicating mechanisms converting unsuitable metabolites into utilisable intermediates.

For maximum enzymic adaptation, it is often sufficient simply to expose the organism to a suitable concentration of substrate, but in other cases the necessity for accessory factors has been demonstrated. Tyrosine decarboxylase was found to be produced in optimal amounts only when pyridoxin and nicotinic acid as well as tyrosine were added to a basal medium (Bellamy and Gunsalus, 1944). The significance of this observation became evident later when pyridoxin was shown to act as coenzyme for amino acid decarboxylases (Gunsalus and Bellamy, 1944*a*). The necessity for nicotinic acid in this adaptation may also be related to its coenzyme function.

Dubos (1940) suggests that although production of new enzymes may occur in the absence of cell division, it must always involve synthesis of new protoplasm. The adaptation of *Saccharomyces cerevisiæ* to galactose fermentation has been found to be dependent, in the absence of other carbohydrate, on the oxidation of galactose itself, which probably provided the necessary energy for enzyme adaptation (Spiegelman, 1945*b*; Reiner and Spiegelman, 1947; Spiegelman, Reiner and Cohnberg, 1947). Adaptation has not been observed where metabolism is not possible. An interesting suggestion as to the origin of adaptive enzymes has been made by Monod (1943, 1944, 1945) and by Spiegelman (1946). These authors have obtained evidence that many enzymes concerned with the metabolism of chemically-related substrates derive from a common pre-enzyme. Some substrates may have greater affinity for the pre-enzyme and will be able to displace other substrates. This principle is seen at work in the adaptation of yeast to various sugars. In the absence of an exogenous source of nitrogen, adaptation to galactose lowers the glucozymase content of yeast cells. If a culture fully adapted to maltose is adapted to galactose, the increase in its galactozymase activity is associated with a sharp drop in maltase activity (Spiegelman, 1946).

Ease of adaptation varies considerably and in some cases actually requires conditions compatible with cell division. The amino acid decarboxylases of *Escherichia coli* are formed optimally only under conditions when cell division can occur,

in the presence of suitable amino acids, and at a *p*H below 5. Here we seem to be close to the borderline, possibly only a convenient mental borderline, between adaptation in response to environment and selection of variants most suited to the environment.

Variation by selection.—The process of variation by selection necessarily involves cell division, and usually becomes evident only after a period of training during which the organism is repeatedly subcultured in an altered and carefully-controlled environment. Variation by selection is, as we have said, a relatively permanent feature. The acquired characteristic requires at least several generations to be lost, and in many cases persists indefinitely after the organisms are returned to their original environment. When variation occurs in response to an environmental stimulus, it is often extremely difficult to decide whether the observed change is an induced response to an external stimulus, or selection of a spontaneous variant which can outgrow the parent under the changed conditions. The two effects can only be distinguished with certainty when the change can be shown to occur spontaneously and independently of the stimulus. The persistence of change after withdrawal of the stimulus does not necessarily imply that the acquired characteristic was selected rather than induced.

Variation may take many forms. Alteration of shape, size, virulence, colonial morphology, antigenicity, permeability, fermentative reactions, nutritive requirements and resistance to drugs have all been recorded. In many cases the altered character is associated with a change in the enzymic make-up of the cell.

As early as 1907, Massini isolated a coliform bacillus, which at first failed to ferment lactose, but which, on culture in a lactose-containing agar medium, gave rise to a variant capable of fermenting lactose. On repeated subculture the variant did not lose its new character. The non-lactose-fermenting strain when grown on lactose media showed a constant capacity for producing lactose-fermenting variants. This instability was not due to an impure strain, because a culture derived from a *single* non-lactose-fermenting cell

behaved in exactly the same way (Kowalenko, 1910). Massini suggested for this strain the name *Bacterium coli mutabile*. Lewis (1934) showed that cultures of this organism contained a constant proportion of variant cells (1 in 10^5) which were capable of fermenting lactose irrespective of the presence of lactose in the culture medium; in other words, the variation was not induced by the environment. The relatively constant occurrence of the variant and the inherited character of the variation would be regarded, in a higher organism undergoing sexual reproduction, as a genetic mutation. In the absence of any knowledge of sexual reproduction in bacteria, it is not possible to term any such hereditary change a *mutation* in its strict sense, but evidence is accumulating, some of which will be referred to later, that a type of change can occur in bacteria which is similar to mutation and to which the word mutation has to be applied for want of a better term.

Deere (1939) suggested that the difference between lactose-fermenting cells of *B. coli mutabile* and the non-lactose-fermenters was chiefly a difference in permeability to lactose; treatment of the non-lactose-fermenting cells with acetone or antiseptics apparently altered the cell permeability, and enabled lactose fermentation to take place. This explanation of the difference between lactose-fermenters and non-lactose-fermenters is, like all explanations dependent upon a permeability hypothesis, difficult to disprove. Recent evidence suggests that the lactase which is demonstrable as a result of treatment with acetone is not the enzyme concerned with lactose metabolism in intact viable lactose-fermenting cells (Lwoff, 1946; Monod and Audureau, 1946; Spiegelman, Reiner and Cohnberg, 1947). In many cases, training or selection involves an actual change in the metabolic capacities of the cell owing to the development of new enzymes. The extracellular proteolytic enzyme of *Clostridium histolyticum* subcultured repeatedly on gelatin is capable of hydrolysing gelatin but not casein (Kocholaty and Weil, 1938). If the organism is repeatedly cultured on a casein medium it eventually develops an enzyme capable of hydrolysing casein but not gelatin.

Alteration of the cellular metabolic processes must also be involved in the training of organisms to grow without certain essential metabolites. Fildes, Gladstone and Knight (1933) trained an " exacting " strain of typhoid bacillus, which was normally dependent upon added tryptophan for growth on a synthetic medium, to grow on ammonia as the sole source of nitrogen. Training consisted of serial subculture into synthetic media containing decreasing amounts of tryptophan, and the fully-trained cells were found to be capable of synthesising tryptophan from ammonia. The dysentery bacillus was trained in the same way to synthesise nicotinamide which it had previously required as a constituent of the medium (Koser and Wright, 1943). *Propionibacterium pentosaceum* can be trained to dispense with thiamin, and, in the process, to develop a capacity for thiamin synthesis (Silverman and Werkman, 1939*a*). In each case it is difficult to distinguish induction of an altered metabolic pattern from selection of spontaneous variants, but, as already noted (p. 101), the term " training " should not be taken to imply an induced response. Training involves a population, not an individual, so that selection of spontaneous variants may explain many examples of nutritional training.

Drug resistance—historical

Before the end of last century, bacteria and protozoa were known to be capable of being trained to grow in concentrations of toxic agents which would prevent all growth of untrained organisms. Most of the early work was limited to the recording of observations, and little attention was paid to the biochemical or clinical significance of the process. The first demonstration of the capacity of adaptation to poisons in bacteria was made by Kossiakoff (1887) by serial subculture in media containing antiseptics. Two years later, Massart (1889) observed that flagellates could be trained to resist what would normally be a toxic concentration of sodium chloride. Abbott (1891) cultivated strains of *Staph. aureus* which could withstand exposure to 1/1000 mercuric chloride for twenty minutes,

although his original culture failed to grow after only five minutes' exposure. Davenport and Neal (1895) developed strains of protozoa capable of growing in normally toxic concentrations of mercuric chloride or quinine by exposing the organism to sublethal concentrations of those drugs. Danysz (1900) reported the isolation of a *B. anthracis* variant which could grow in solutions of arsenic five times as concentrated as those which inhibited the original strain.

In 1908 Ehrlich and his school made the fundamental discovery that treatment of trypanosome-infected animals with sub-optimal doses of trypanocidal drugs led to the development of drug-resistant infections. This observation stimulated an interest in drug-resistant protozoa which for a time relegated bacterial drug resistance to the background. As investigation of the two fields followed different lines, they will be considered separately and protozoal resistance will be discussed later.

Scattered observations on the development of resistance to antibacterial agents continued to be made, but these were without clinical significance since the antibacterial agents were only suitable for use *in vitro*. Morgenroth and Kaufmann (1912) were, however, able to show that resistant strains of pneumococci could be produced *in vivo* by treatment of pneumococcal infections of mice with ethylhydrocupreine.

The relatively permanent character of bacterial resistance to drugs was demonstrated by Seiffert (1911), who noted that a strain of *Escherichia coli* which had been rendered resistant to malachite green by adding the dye in gradually increasing concentrations to successive cultures, was still resistant after seventy-five transfers in normal media. Strains of cholera vibrio resistant to various dyes such as methylene blue and trypaflavine also maintained their resistance through subculture in the absence of dye (Shiga, 1913). Ricket, Bachrach and Cardot (1922) studied in some detail the acquired tolerance of lactic acid bacteria for certain metallic poisons, and noted that resistance was specific for a given poison. When lactic acid bacteria rendered resistant to potassium chloride were

grown in a potassium chloride-free medium together with the parent non-resistant strain, the resistant strain was rapidly and completely eliminated by the parent strain (Cardot and Laugier, 1923).

When an organism rendered resistant to one drug becomes at the same time resistant to a second unrelated drug, it is said to exhibit *cross resistance*. Cross resistance in bacteria was demonstrated by Jungeblut (1923) with a variety of organisms and with such drugs as mercuric chloride, optochin, rivanol, trypaflavine and methylene blue. *Escherichia coli*, trained to be resistant to the mercuric ion, was found to be resistant also to other cations such as barium, calcium, iron and copper (Borman, 1932).

Kappus (1930), from a study of the resistance of *E. coli* to malachite green, concluded that development of resistance only occurred when bacteria were actively growing in the presence of toxic agents. *Staph. albus* resistance to acriflavine, which was rapidly lost in a drug-free medium, was developed by exposure to the drug for only six or eight hours (Burke and Ulrich, 1928; Burke, Ulrich and Hendrie, 1928). These authors suggested that the changes associated with this resistance were purely an adaptation to a new environment, and in no way associated with changes in life cycle, selection of variants or mutation.

With the advent of the sulphonamides and the introduction of clinical bacterial chemotherapy, the recognition that bacteria could easily acquire resistance to sulphonamides (Maclean, Rogers and Fleming, 1939) provided the stimulus for intensive research on drug resistance in bacteria. It is now evident that most bacteria can acquire resistance to many chemotherapeutic drugs and antiseptics, with the exception of phenols and detergents. The antibiotics such as penicillin and streptomycin also give rise to resistant strains (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941; Miller and Bohnhoff, 1946). Increasing dependence on chemotherapy for the clinical control of infection therefore results in drug resistance assuming an increasingly important position as a practical problem.

Development of drug resistance in bacteria

Emphasis should be laid on the fact that, quite apart from acquired resistance, different bacterial species and strains possess varied inherent susceptibilities to drugs. In addition to the well-known resistance of Gram-negative organisms to sulphonamides and penicillin, marked differences occur

TABLE 16

Bacteriostatic action of antibiotics on different strains of staphylococcus

± = partial growth
\+ = full growth
/ = no growth

Antibiotic	Concentration of Antibiotic	Strain of Staphylococcus							
		1725		CH		2019		3	
		24 hours	96 hours	24 hours	96 hours	24 hours	96 hours	24 hours	96 hours
Penicillin	1·0 μg./ml.	/	+	/	/	/	/	/	/
	0·1 μg./ml.	+	+	/	/	/	/	/	/
Streptothricin	20 mg./100 ml.	/	/	/	±	/	/	/	+
	10 mg./100 ml.	/	+	+	+	/	/	+	+
Tyrothricin	2·5 mg./100 ml.	/	/	/	/	/	/	/	/
	0·8 mg./100 ml.	/	/	+	+	/	/	/	/
Control	...	+	+	+	+	+	+	+	+

From Neter (1945).

between drug susceptibilities of different strains of the same Gram-positive organism. This is illustrated by Table 16 where the action of various antibiotics on several strains of staphylococcus is displayed (Neter, 1945).

The response of bacteria to various drugs is, as we shall show, a complex of different factors, and the inner mechanism varies with species and with drug so that no one explanation of drug resistance can cover more than a few individual cases.

Resistance can be developed both *in vitro* and *in vivo* by contact of the bacterium with sub-lethal concentrations of drug through several successive generations. Development of resistance is a gradual process, but a slight increase in resistance is usually observable after one subculture in a medium containing a drug. Table 17 gives figures for the development of resistance to sulphapyridine in the pneumococcus (Schmidt and Sesler, 1943).

TABLE 17

Conversion of a sulphapyridine-sensitive strain of pneumococcus to a resistant strain

Number of previous transfers in sulphonamide medium	Number of colonies per plate with x mg. per cent. of sulphapyridine in medium										
	x=0	0·3	0·6	1·25	2·5	5·0	10·0	20·0	40·0	80·0	144
0 (Control—beef heart broth)	188	192	159*	0	0	0	0				
1	136	130	112	114	125*	0					
2	99			131	124	83*	1	0			
3	296					258	280	128	0	0	
4	174						130	96	90	70*	0
5	127							130	109	74*	11*
6	269								232	162*	30*
7	332								340	316	200*
8	189								205	207	104*

* Colonies abnormally small.

From Schmidt and Sesler (1943).

Considerable variation in the sensitivity of individual cells of a single normal culture has been demonstrated by various workers with sulphonamides and other drugs (Schmidt and Sesler, 1943; Horsfall, 1942; Demerec, 1945*b*; Doudoroff, 1940; Severens and Tanner, 1945).

In some cases, the final degree of resistance developed was dependent upon the concentration of drug in which subculture was carried out. The tolerance of *Bact. lactis aerogenes* (*Aerobacter aerogenes*) to proflavine was approximately equal to

the concentration used in the training medium (Davies, Hinshelwood and Pryce, 1945). A strain of *Escherichia coli* subcultured on media containing 100 μg./ml. of sulphathiazole was found to be highly resistant to this drug, whereas the same strain subcultured on 2·5 μg./ml. sulphathiazole media developed only a low degree of resistance (Kirby and Rantz, 1943). On the other hand, Strauss, Dingle and Finland (1941), also using *E. coli*, found that the concentration of sulphonamide in the medium was of little importance and that resistance to high concentrations of drug could be developed by repeated transfer in media containing little drug. Hinshelwood (1946) found that *B. lactis aerogenes* trained on low concentrations of sulphonamide was likewise resistant to high concentrations of sulphonamide.

It seems fairly certain that each organism and each strain can develop a maximum degree of resistance beyond which it is impossible to go by further training. When resistance has reached this point the organism is usually termed fully resistant; this term must not be accepted in its literal sense, since the organism is still affected by concentrations of drug above the training limit. The maximum resistance capable of being developed varies greatly with different organisms; for example, three strains of staphylococcus rendered fully-resistant to penicillin possessed from 1000 to 6000 times normal resistance, a strain of streptococcus could only be made thirty times more resistant than the parent strain, while pneumococcus types I and II could only develop six times the resistance of the parent strain (McKee and Houck, 1943).

The rapidity with which resistance is acquired also varies greatly with different organisms and different drugs. Table 18 indicates the rate of development of resistance in a single strain to four different drugs (McIntosh and Selbie, 1943).

Once resistance has been acquired, it is often, but by no means always, a fairly permanent characteristic of the strain, and may survive numerous transfers in the absence of drug. In the case of sulphonamide resistance, partially-trained organisms were found to revert to the non-resistant state on subculture more readily than fully-resistant organisms

(Dettwiler and Schmidt, 1940 ; Schmidt, Sesler and Dettwiler, 1942 ; Davies and Hinshelwood, 1943). This no doubt accounts for some of the reports that sulphonamide resistance is not permanent. A curious case of loss of sulphonamide resistance has been reported by Davies, Hinshelwood and Pryce (1944). A sulphonamide-resistant strain of *Bact. lactis aerogenes* was subcultured in a medium containing proflavine but no sulphonamide. Under these conditions the organism acquired

TABLE 18

Development of drug resistance in Staph. pyogenes

Successive subcultures	Highest concentration of drug permitting growth			
	Propamidine	Proflavine	Quindoline Methochloride	Penicillin (Standard Solution = 200 units/ml.)
1	1/80,000	1/160,000	1/80,000	1/20,000
2	1/40,000	1/80,000	1/40,000	1/10,000
3	1/20,000	1/80,000	1/20,000	1/10,000
4	1/10,000	1/40,000	1/10,000	1/5000
5	1/10,000	1/40,000	1/10,000	1/2500
6	1/10,000	1/20,000	1/10,000	1/2500
7-8	1/10,000	1/20,000	1/10,000	1/2500
9	1/10,000	1/20,000	1/10,000	1/1250
10	1/5000	1/10,000	1/10,000	1/1250
11	1/5000	1/10,000	1/10,000	1/625
12-13	1/5000	1/10,000	1/10,000	1/625
14	1/2500	1/10,000	1/10,000	1/312
15-20	1/2500	1/10,000	1/10,000	...
21	1/1250	1/10,000	1/10,000	...

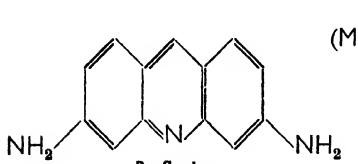
From McIntosh and Selbie (1943).

resistance to proflavine but lost a considerable degree of sulphonamide resistance. The converse effect was not observed ; a proflavine-resistant strain after subculture in media containing sulphonamide but no proflavine became fully resistant to both sulphonamide and proflavine.

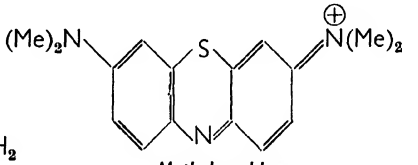
Specificity of resistance

An organism rendered resistant to one drug is often found to have acquired at the same time cross resistance to chemically-related drugs and sometimes also to chemically-unrelated

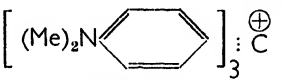
drugs. *B. lactis aerogenes* rendered resistant to proflavine was also resistant to methylene blue, and *vice versa*. Methylene blue and proflavine inhibited the growth of this organism in an identical manner, and the process of development of resistance followed the same course with both compounds. These observations suggest that the two drugs have a similar mode of action (Davies, Hinshelwood and Pryce, 1944).



Proflavine



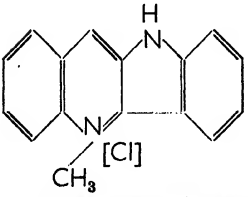
Methylene blue



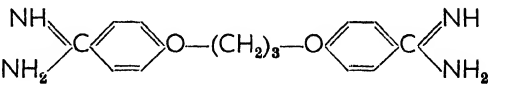
Crystal violet

Crystal violet, which is not related structurally to the other two dyes, can also give strains cross-resistant to proflavine and methylene blue, but the degree of cross resistance is not so great. Sulphanilamide-resistant *B. lactis aerogenes* showed no cross resistance with any of the three dyes.

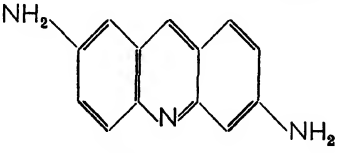
McIntosh and Selbie (1943) investigated the cross resistance of *Staph. pyogenes* using quindoline methochloride, penicillin,



Quindoline methochloride



Propamidine



2 : 7-Diaminoacridine

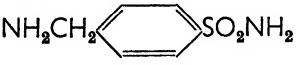
sulphonamides, acridines and amidines. Penicillin-resistant strains showed no cross resistance for any of the other drugs ;

proflavine, quindoline methochloride and propamidine showed cross resistance to one another. Resistance to 2 : 7-diaminoacridine could not be developed, but cultures grown in the presence of this drug were resistant to propamidine and proflavine. Cross resistance among antibiotics is the exception rather than the rule, but streptomycin-resistant *Proteus vulgaris* showed some cross resistance to streptothricin (Waksman, Reilly and Schatz, 1945).

Two types of *Salmonella* (*pullorum* and *schotmülleri*) and *Eberthella typhosa* could be rendered resistant either to sodium chloride, mercuric chloride or copper sulphate, but each resistant strain was specific, no cross resistance from one inorganic salt to another being found (Severns and Tanner, 1945). On the other hand, as already mentioned, *Escherichia coli* developed cross resistance to barium, calcium, iron and copper ions when made resistant to mercury or magnesium (Borman, 1932).

It will be evident, from what has been said, that the development of a resistant strain can be a highly specific or a quite unspecific process. It may be a specific response to a drug, or it may be selection of a naturally-occurring variant which is less susceptible to injury by all chemicals. Schnitzer, Camagni and Burk (1943) found that a small-colony-variant of *Staph. albus*, isolated by growing the organism in a penicillin medium, was also resistant to such unrelated substances as methyl violet and barium chloride.

The character of sulphonamide resistance offers a direct contrast to such non-specific effects. Numerous workers have found that resistance to one sulphonamide only implies resistance to other related sulphonamides which are antagonised by *p*-aminobenzoic acid (P.A.B.A.) ; there is no cross resistance to such a closely related drug as marfanil



which is not antagonised by P.A.B.A. It would seem that within the group of sulphonamides antagonised by P.A.B.A., many organisms adopt a common mechanism for the development of full resistance. Cases have been reported, however,

where resistance to one such sulphonamide has not been carried over to another (Cooper and Keller, 1943; Colebrook, 1943; Strauss *et al.*, 1941). There are two possible explanations of such discrepancies. First, the degree of resistance to a sulphonamide is correlated with the bacteriostatic potency of that sulphonamide. For example, organisms made resistant to sulphanilamide at a concentration of 100 μg. per ml. are not resistant to the same concentration *by weight* of sulphadiazine, but rather to an equivalent *bacteriostatic* concentration which is less than 2·5 μg. per ml. Because of this great difference in potency, it is possible that cross resistance from a weak to a highly-bacteriostatic sulphonamide can easily be missed (Kirby and Rantz, 1943). Cross resistance may also be missed owing to failure to establish resistance fully before testing for cross resistance. Although fully-resistant strains of *Bact. lactis aerogenes* are almost equally resistant to all sulphonamides, this does not hold in a partly-trained strain. During training, specific resistance to the drug which is being used for training develops more quickly than resistance to other sulphonamides (Davies and Hinshelwood, 1943). In some organisms, even the mechanism of development of full resistance may vary according to the sulphonamide used.

Mechanism of drug resistance

An organism may display resistance to a harmful agent through being able to prevent access of that agent to the interior of the cell. Obviously then, selection, over a number of generations, of cells with decreased permeability to a drug could result in a resistant strain. Such a mechanism is probably involved in the development of trypanosome strains resistant to organic arsenicals (see p. 292). The spirochæte of relapsing fever can become resistant to neoarsphenamine or solganal; its normal form has been found to absorb arsenic or gold from solutions of these drugs while the resistant form does not (Fischl and Singer, 1934 *a* and *b*; Fischl, Kotrba and Singer, 1934). Jancsó and Novák (1935) claimed, however, that some non-resistant strains of *Spirochæta usbekistanica* which were readily killed by gold drugs failed to bind gold.

Little evidence has appeared to show whether drug resistance in bacteria is related to change of cell permeability. The lack of evidence is no doubt due to experimental difficulties, but as tracer elements become available these difficulties should be more readily overcome. Yegian and Budd (1945) found that sulphonamide-resistant *Mycobacteria* were no less permeable to sulphonamides than the parent non-resistant strains. In few cases of bacterial drug resistance has it been possible to exclude entirely the possibility of some permeability change, but in many cases development of resistance is undoubtedly associated with alteration of enzymic make-up of the cell.

We have already seen how easily adaptation may arise in response to a change of environment, and also how selection can encourage growth of variants which are more suited to a new environment than the original strain. Since we have evidence that many drugs act by interference with enzyme systems, it is reasonable to assume that drug resistance may be acquired by alteration of the enzymic constitution of the cell. We can envisage several ways in which such variation might occur—

(1) by increased production of an essential metabolite or drug antagonist ;
(2) by development of a non-sensitive alternative pathway for the synthesis or degradation of an essential metabolite ;
(3) by development of the capacity to detoxicate a drug either by conjugation or by oxidative destruction.

Increased production of essential metabolite

The bacteriostatic action of sulphonamides can be antagonised by addition of P.A.B.A. to the culture medium, and the available evidence suggests that sulphonamides act by competition with P.A.B.A. in an essential enzyme system. In other words, these drugs reduce the effective concentration of P.A.B.A. available to the cell. An organism might adapt itself to the presence of sulphonamide by an enzymic variation which permitted synthesis of increased amounts of P.A.B.A. Strains possessing a high degree of natural resistance might

also be expected to show an inherited capacity for the production of large amounts of P.A.B.A., unassociated with training to the presence of drug in the growth medium.

In some cases at any rate, the above explanation may hold. Landy and Gerstung (1944) found that in 175 strains of *Neisseria gonorrhœæ* isolated clinically, response of the patients to sulphonamides closely paralleled production of P.A.B.A. in culture media by the isolated organisms. Cultures isolated from patients who did not respond to sulphonamide therapy

TABLE 19

Comparative assay of sulphathiazole resistance and of p-*aminobenzoic acid production by different strains of staphylococcus*

Strain	Sample	P.A.B.A., μg./ml.	Minimum concentration sulphathiazole inhibiting growth, in mg./100 ml.
7	Cells	1·7	Less than 1·0
	Filtrate	1·8	...
14	Cells	1·1	Less than 1·0
	Filtrate	1·1	...
7c	Cells	130	50
	Filtrate	130	...
14c	Cells	57	75
604	Cells	200	200
605	Cells	170	250

From Spink, Wright, Vivino and Skeggs (1944).

were resistant *in vitro* to the drugs and produced large amounts of P.A.B.A. in culture fluid; while strains isolated from patients who were readily cured were sulphonamide-sensitive and produced relatively little P.A.B.A. in culture. Some relation has been found also between high P.A.B.A. production and sulphonamide-resistance of various strains of staphylococcus as indicated in Table 19 (Spink, Wright, Vivino and Skeggs, 1944). With two strains of pneumococcus (types I and VIII) obtained from patients who did not respond to sulphadiazine treatment, no increased production of P.A.B.A. or any other sulphonamide antagonist could be found, but

another resistant strain (type I) produced large amounts of a sulphonamide inhibitor (Tillet, Cambier and Harris, 1943).

It would appear then that some naturally-resistant strains do produce increased amounts of P.A.B.A., but that others do not. The same variable effect has been shown with organisms trained *in vitro*. *Staph. aureus* made resistant to sulphonamides produced 3·3 μg. of P.A.B.A. per ml. of culture medium, while the parent non-resistant strain produced under the same conditions only 0·033 μg. of P.A.B.A. per ml. (Landy, Larkum, Oswald and Streightoff, 1943). In all these cases P.A.B.A. was not demonstrated by chemical isolation but by colorimetric or biological assay. Sevag (1946) has suggested that the arylamine estimated may not have been P.A.B.A.

Strains of *Escherichia coli*, *Vibrio cholerœ*, *Shigella dysenteriœ* and *Diplococcus pneumoniœ*, trained to resist sulphonamides, all failed to produce greater amounts of P.A.B.A. (Landy *et al.*, *loc. cit.*; Housewright and Koser, 1944). This failure to demonstrate increased production of P.A.B.A. by resistant strains does not, however, exclude participation of P.A.B.A. in the resistance mechanism. Link (1943) found that the addition of P.A.B.A. at a level of 1 μg. per ml. of culture medium antagonised the inhibitory effect of sulphathiazole on a resistant strain of staphylococcus, while this amount had no effect in protecting non-resistant strains. A tenfold increase in P.A.B.A. concentration prevented sulphonamide bacteriostasis in both cases.

To sum up, the evidence for the participation of the P.A.B.A. system in sulphonamide resistance in some organisms is fairly convincing, but not absolutely indisputable. It is possible that the observed production of P.A.B.A. may be a secondary effect of training rather than the cause of resistance, since in many organisms increased resistance does not appear to be associated with increased production of P.A.B.A.

As indicated in the discussion of thiamin antagonists, the development of pyrithiamin resistance in yeast did not depend upon production of excess thiamin, and the amount of thiamin synthesised by naturally-resistant strains of micro-organisms was insufficient to account for their ability to grow in high

concentrations of pyrithiamin (Woolley and White, 1943*b*; Woolley, 1944*c*).

Nutritional exactitude

It will be remembered that certain bacteriostatic agents other than the sulphonamides are also believed to act through a process of competitive inhibition of the synthesis or utilisation of an essential metabolite. Thus, pantoyl-taurine is an antagonist of pantothenic acid. McIlwain (1943*a*) found that development of resistance by *Corynebacterium diphtheriæ* to pantoyl-taurine was associated with a decrease in the nutritional exactitude of the organism. Strains of this organism vary greatly in their pantothenic acid requirements, from independence of all sources of the metabolite to complete dependence on presynthesised acid. All fourteen strains of *C. diphtheriæ* examined by McIlwain required pantothenic acid for growth, but only some of the strains required preformed pantothenic acid; other strains were able to synthesise their own requirements provided the medium contained the β-alanine portion of the molecule. Of the second group, strains which were relatively resistant to pantoyl-taurine could grow on a medium containing less β-alanine than the more sensitive strains (see Table 20). Three of the more sensitive strains, when trained to resist pantoyl-taurine by growth in the presence of the drug, were found to have acquired ability to grow on lower concentrations of β-alanine. Change in resistance to pantoyl-taurine could also be developed by a process of direct nutritional training. Strains requiring a high concentration of β-alanine were subcultured in media containing gradually decreasing amounts of β-alanine, and as a result, variants were obtained which could grow readily on low concentrations of β-alanine. In other words, they were less nutritionally-exacting than the original strain. The same effect could be achieved by starting subculture in media containing small concentrations of β-alanine and sufficient pantothenic acid to permit growth, and transferring to media containing decreasing concentrations of pantothenic acid. The final variant in either case was

TABLE 20

Effect of training on pantoyl-taurine sensitivity and its relation to ability to grow in decreased concentrations of β-alanine. Organism C. diphthériæ. (McIlwain, 1943*a*).

P.A. = pantothenic acid. P.T. = pantoyl-taurine.

Strain	Method of training strains	Minimum molar concentration of β-alanine producing		$\frac{PT}{PA}$ ratio. Minimal for inhibition of growth during four days
		Growth in one to two days	Growth in three to four days	
G.I.	High concentration P.A.	None at 10^{-5}	None at 10^{-5}	200-500
	Decreasing concentration P.A.	Trace at 10^{-5}	2×10^{-6}	2,000
	Increasing concentration P.T.	10^{-6}	10^{-7}	10,000
	Decreasing concentration β-alanine	10^{-6}	10^{-7}	10,000
GW 1	Untrained	5×10^{-6}	2×10^{-6} to 4×10^{-7}	4,000-5,000
	High concentration P.A.	None at 10^{-5}	Trace at 10^{-5}	800-1,000
	Decreasing concentration P.A.	4×10^{-7}	No addition *	>20,000
	Decreasing concentration β-alanine	10^{-6}	No addition *	>20,000
	Increasing concentration P.T.	4×10^{-7}	No addition *	>20,000
351	High concentration P.A.	None at 10^{-5}	10^{-5}	1,000-2,000
	Decreasing concentration β-alanine	2×10^{-6}	4 to 8×10^{-7}	20,000
	Increasing concentration P.T.	10^{-5}	4 to 8×10^{-6}	100,000
1111	No training	2×10^{-6}	No addition *	>100,000
	High concentration P.A.	10^{-5}	2×10^{-6}	2,000
	Increasing concentration β-alanine Increasing concentration P.T.	No addition *	No addition *	>100,000

* The basal medium used was a hydrolysed casein medium which contained β-alanine at a concentration of 5×10^{-3}M.

considerably more resistant to pantoyl-taurine than the parent strain. Two of the more resistant of the naturally-occurring strains (GW 1 and 1111), when subcultured in the presence of large amounts of pantothenic acid, became more nutritionally-exacting and, at the same time, less resistant to pantoyl-taurine.

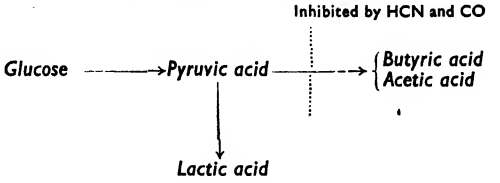
It is evident from these results that adaptation to grow on a medium containing β-alanine but no pantothenic acid is directly connected with acquisition of resistance to pantoyl-taurine. The results imply that, in a trained strain capable of growth on pantothenic acid free media, an enzyme system exists for the synthesis of pantothenic acid from β-alanine. The possibility that pantoyl-taurine resistance was due to a change in cell permeability was excluded, as similar quantities of intracellular pantothenate were found in both parent strain grown in presence of pantothenate, and in resistant strain grown without pantothenate.

By a similar process of nutritional training, staphylococci were made independent of most amino acids. The non-exacting trained strain was less susceptible to inhibition by the related α-aminosulphonic acid than the parent strain (McIlwain, 1941*b*).

Alternative metabolic pathways

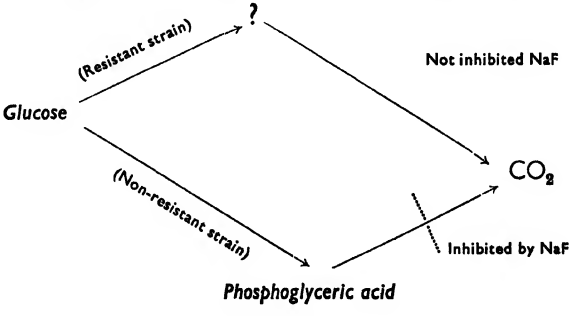
Many organisms are known to be capable of utilising a given metabolite by more than one route. It is not possible to say if this capacity developed originally as an insurance against interference with normal food supply or toxic agents, but there is evidence that in the presence of certain cell poisons alternative metabolic pathways may be called into play. Kubowitz (1934) found that the end products of glucose (or pyruvate) fermentation by *Clostridium butyricum* were altered by the presence of cyanide or carbon monoxide. In the absence of poison, the main products were acetic and butyric acids, but when poison was present mainly lactic acid was produced. Apparently carbon monoxide and cyanide inhibited the enzyme systems essential for production of butyrate and acetate from pyruvate, but did not inhibit an enzyme also present which converts

pyruvate to lactate. A similar effect on glucose metabolism was produced by growing *Clostridium welchii* in an iron-



deficient medium (Pappenheimer and Shaskan, 1944). Iron is, of course, essential for the functioning of those enzymes which are susceptible to inhibition by carbon monoxide or cyanide, so that the effects of iron deficiency and of cyanide inhibition might be expected to be alike. It is apparent then, that in the presence of an enzymic poison, or in the absence of an essential component of an enzyme system, an organism may survive by utilisation of an alternative enzyme system which performs the same function by different means (*cf.* p. 113).

The use of an alternative metabolic pathway during development of resistance to sodium fluoride was indicated by the results of Wiggert and Werkman (1939). *Propionibacterium pentosaceum* grown anaerobically in the presence of glucose degraded both glucose and phosphoglyceric acid (a normal intermediate) to carbon dioxide. The fermentation was inhibited by sodium fluoride which is known to inhibit breakdown of phosphoglyceric acid (p. 161). A strain was developed resistant to sodium fluoride when grown on glucose medium, and unable to utilise phosphoglyceric acid as a substrate. The results suggest that the mechanism of resistance might be represented diagrammatically as below :

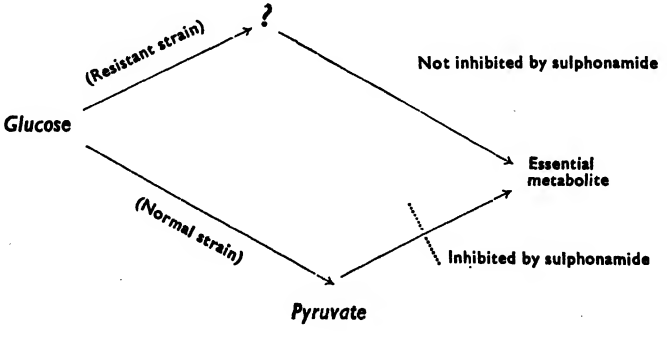


Evidently, an alternative method of glucose fermentation was developed which did not utilise phosphoglyceric acid as an intermediate.

It is difficult to exclude the possibility that the difference between resistant and non-resistant strains is a difference in permeability, since the same experimental result would be observed if the resistant strain had become impermeable to both sodium fluoride and phosphoglyceric acid (Lipmann, 1941). Soaking of the resistant cells overnight in fluoride prior to the addition of glucose had, however, no effect on fermentation.

Evidence for the utilisation of alternative metabolic pathways by strains of micro-organisms resistant to chemotherapeutic drugs is necessarily indirect, since in no case do we know with certainty the complete nature of the metabolic process inhibited by a drug. Indirect evidence has been collected, however, which suggests that many cases of drug resistance do involve a change in the metabolic pathways of the cell.

Staph. aureus, trained until resistant to sulphonamides in the presence of glucose, was not resistant when grown on a medium in which glucose was replaced by pyruvate (Sevag and Green, 1944*b*). Such a result suggests that sulphonamides blocked some step in the normal metabolism of glucose through pyruvate, and that the resistant organism developed an alternative metabolic process which by-passed pyruvate. Diagrammatically the effect could be represented as below :



Repeated subculture of resistant organisms in a glucose-free medium resulted in a strain resistant to sulphonamides even in the absence of glucose.

These results indicate that the nature of the medium has an important influence on the biochemical characteristics developed during selection of a resistant strain. Strains of *Escherichia coli*, trained to a high degree of sulphonamide resistance on peptone media, were no more resistant to sulphonamides than the parent strain when grown on a salt-glucose medium (Strauss, Dingle and Finland, 1941; Harris and Kohn, 1943). Evidently the organism develops a type of resistance based on the most readily-available metabolite, and if that metabolite is omitted from the medium the organism has to be re-trained before becoming drug-resistant on the simplified medium. Results of this type could hardly be explained on a permeability basis, since one would have to assume that a resistant organism was impermeable to a drug only in the presence of the metabolites used in the training media, or that during training it became impermeable to all metabolites except those available in the training media. Sevag summed up the function of the medium in drug resistance by postulating that an organism can more readily adapt itself to the presence of substances which interfere with certain of its usual metabolic functions if its environment contains other substances which can be utilised for the development of alternative mechanisms of synthesis. Furthermore, organisms may differ innately in their ability to utilise substances in the environment or to synthesise them. Thus they may differ in their ability to alter their metabolic activities in the presence of conditions unfavourable to their usual type of metabolism.

An example of specific change in growth factor requirements during development of resistance by *Escherichia coli* was reported by Kohn and Harris (1942). A strain resistant to sulphanilamide was developed by training in synthetic media containing methionine and increasing amounts of sulphonamide; during training, methionine became an essential growth factor for the strain which had originally been capable

of growing without it. A resistant strain trained in the absence of methionine did not require it as a growth factor. It has been suggested that methionine synthesis is inhibited by sulphonamides (Chapter V). If this is so, then one must assume that the strain which developed sulphonamide resistance in the absence of methionine had evolved a method for the synthesis of methionine by a sulphonamide-resistant route, or had dispensed altogether with the use of methionine. The strain rendered resistant to sulphanilamide in the presence of methionine did not develop any such mechanism; its normal mechanism for methionine synthesis was, however, unusable in the presence of sulphanilamide and the variant became dependent upon a supply of methionine in the medium. Analysis of "mutant" strains has shown the nature of one metabolic block which occurs in the sulphonamide-resistant methionine-exacting strain. *E. coli* is normally able to synthesise its entire requirements of sulphur-containing substances from inorganic sulphur. The route of synthesis of methionine from sulphate is probably as follows :—

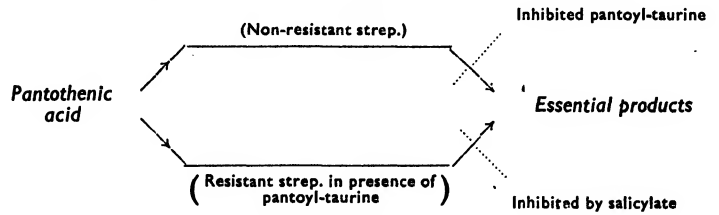
$$SO_4'' \longrightarrow SO_3'' \longrightarrow S \longrightarrow \text{cysteine}$$

$$\text{methionine} \longleftarrow \text{homocysteine} \longleftarrow \text{cystathionine}$$

The point of enzyme loss in the variant made resistant to sulphonamide in the presence of methionine was indicated by the observation that a strain prepared in this way was unable to convert homocysteine to methionine (Lampen, Roepke and Jones, 1947).

It will be remembered that pantoyl-taurine-resistant strains of *Corynebacterium diphtheriæ* did not require added pantothenic acid but were able to synthesise their own requirements from β-alanine. Experimentally-produced resistant strains of *Strep. hæmolyticus* still require added pantothenate for growth. These strains are said to be susceptible to inhibition by salicylate. It would appear that "strep.", unlike "dip.", is incapable of variation involving development of an enzyme system for synthesis of pantothenic acid. In the presence of pantothenate the "strep." may develop a pantoyl-taurine-

resistant mechanism for the utilisation of added pantothenate which is possibly susceptible to blocking by salicylate as indicated diagrammatically below (McIlwain, 1943*a*).



A few reports have appeared relating drug resistance to change in enzymic activity. Loss of dehydrogenase activity for glycerol, lactate and pyruvate, but not for glucose, has been found to be associated with the acquisition of sulphonamide resistance in pneumococci (MacLeod, 1939), but of course the loss of individual enzymic activities of this type will no doubt depend largely on the nature of the medium used during training. Pneumococci rendered resistant to penicillin have been found to possess a decreased ability to ferment lactose (Abraham *et al.*, 1941). *Bact. lactis aerogenes*, trained to moderate concentrations of sulphanilamide, proflavine or crystal violet, showed no significant change in dehydrogenase activity, but high concentrations of drugs had measurable effects. Training to sulphonamide, enhanced the catalase activity of cells ; training to proflavine, decreased catalase activity. Training to high concentrations of crystal violet lowered glycerol dehydrogenase activity, but training to proflavine had the opposite effect (Hinshelwood, 1946 ; Cole and Hinshelwood, 1947 ; Davies and Hinshelwood, 1947). These results are particularly interesting in view of the demonstration that training to proflavine may reduce the sulphonamide resistance of a sulphonamide-resistant strain (p. 257).

Enzymic destruction of drug

An account has already been given of the development of pyrithiamin resistance in *Endomyces vernalis*. This resistance was associated with the development of an enzyme which split the pyrithiamin molecule into two non-toxic halves

p. 224). Some penicillin-resistant strains of Staphylococcus also depend for their resistance upon their ability to convert penicillin to inactive products. The enzyme penicillinase which destroys penicillin was first demonstrated in Gram-negative bacilli by Abraham and Chain (1940). Staphylococci isolated from some penicillin-resistant clinical infections have been found to produce considerable quantities of penicillinase (Kirby, 1944 ; Bondi and Dietz, 1945 ; Gots, 1945). Although penicillinase-producing organisms can destroy penicillin, the individual cells are not highly resistant to penicillin and the resistance of a culture depends upon the ability of a large inoculum to destroy penicillin before the drug can act (Luria, 1946). Penicillin resistance of the type examined by Demerec (p. 276) does not depend on destruction of penicillin by penicillinase.

Although other cases of resistance involving development of an enzyme designed to destroy the drug have not been examined in detail, there are indications that a similar mechanism may be at work in some forms of drug resistance. Emerson and Cushing (1946) obtained a sulphonamide-resistant strain of *Neurospora* for which sulphanilamide was a growth factor. Presumably the organism had developed enzymes for the conversion of sulphanilamide into some essential metabolite. The results of Link (1943) suggest a similar process in some resistant strains of staphylococci. Resistance to streptomycin is particularly easily developed. In the meningococcus two types of streptomycin resistance have been distinguished. Type A resistance appeared randomly, and the resistant strain was able to grow on any medium which supported the original strain. Colonies of type B variant differed from the above in that they only developed on media containing more than 40 μg./ml. of streptomycin and, once isolated, required streptomycin in the medium for growth (Miller and Bohnhoff, 1947*a*).

Penicillin resistance and glutamic acid metabolism

The biochemical basis of penicillin resistance in *Staph. aureus* has been studied in detail by Gale (1947*b*). Penicillin

shows remarkably selective toxicity; extremely low concentrations inhibit growth of Gram-positive micro-organisms, but even high concentrations have little effect on Gram-negative organisms or animal cells. We have already indicated that the selective toxicity of sulphonamides is dependent upon the existence in sulphonamide-sensitive cells of a mechanism for the synthesis of pteroylglutamic acid which is absent in sulphonamide-resistant cells. The selective action of penicillin suggests that it also inhibits a biochemical reaction essential to a group of micro-organisms, but inessential to or non-existent in animal cells and Gram-negative bacteria. In searching for metabolic differences between Gram-positive and Gram-negative organisms, Gale and Taylor noted that the two groups differed widely in their amino acid requirements. Many Gram-negative organisms synthesised their amino acids, Gram-positive organisms depended to a large extent on a supply of amino acids in the medium. The passage of amino acids from the medium to the interior of the cell was, in some cases, a passive diffusion process; but glutamic acid, glutamine and histidine were transported across the cell wall by an endergonic metabolic reaction. Such a transport mechanism for amino acids did not exist in Gram-negative organisms (Gale and Taylor, 1946*b*, 1947*b*; Gale, 1947*a*; Taylor, 1947). The recognition that an endergonic biochemical mechanism for amino acid uptake was of special importance in Gram-positive organisms suggested that drugs which showed selective action against such organisms might act by inhibiting amino acid assimilation. Gale and Taylor found that penicillin did, in fact, inhibit uptake of glutamic acid by *Staph. aureus*.

It might easily be argued that the observed inhibition of glutamic acid uptake was the result, and not the cause, of penicillin action. This is an argument which will always arise in study of the mode of action of a drug and it is one which is most difficult to refute. Respiration, glucose oxidation, glucose fermentation and lysine assimilation of penicillin-inactivated cells are all normal, even glutamic acid metabolism within the cell is normal, but viability and glutamic acid uptake

fall together ; which is the cause, which the effect ? Gale and Taylor believe that inhibition of glutamic acid uptake precedes cell death.

Gale (1947*b*) has sought evidence in support of this view in his study of penicillin resistance. He found that when one organism was ten times more resistant than another, then ten times as much penicillin was required to block glutamic acid assimilation in the former as compared with the latter. When *Staph. aureus* was trained by serial subcultivation in increasing concentrations of penicillin, increased resistance to penicillin was accompanied by decreased affinity for the glutamic acid in the medium. It follows that if resistance was pushed to the point where the cell no longer assimilated glutamic acid, the organism would have to change the nature of its metabolism and synthesise an internal supply of glutamic acid. Such a change has been reported (Gale and Rodwell, 1948), but it remains to be seen whether all cases of penicillin resistance (excluding production of penicillinase) can be explained on this basis, or whether ability to synthesise glutamic acid is only one facet of the change involved in development of penicillin resistance.

Origin of resistant forms

In the preceding sections we have shown that the measurable result of the acquisition of drug resistance may be altered metabolism or permeability, but we have neglected the question as to how a resistant strain comes into being.

Attempts to elucidate the gradual increase in resistance of a bacterial culture encounter a basic problem common to all studies of physiological change in large populations. Large numbers of individual cells are always used in the development of resistant strains, so that the resulting biochemical modifications are not measured on single cells, but are the over-all changes in a whole group of individuals. The capacity of an individual cell for adapting itself to an environmental change may be limited, but to this must be added the physiological pliability of the group in terms of the numbers and kinds of variants it is capable of producing. In any given case, the

same end result may be achieved by any one of three mechanisms :—

(1) Selection of existent or spontaneously-occurring variants from a heterogeneous population.
(2) Induction of changed biochemical reactions by contact of cell enzymes with drug, occurring in all members of a homogeneous population.
(3) Selection from a heterogeneous population of those cells which are capable of modification by mechanism (2).

As far as bacteria are concerned, it is inherently difficult to separate these mechanisms, since genetic control over a population is not possible (Spiegelman, 1945*a*). However, it has been possible in some cases to obtain indirect evidence for the origin of resistant forms.

Selection of spontaneous variants

As already mentioned, Lewis (1934) was able to decide that trained strains of *Escherichia coli* (*B. coli mutabile*), capable of fermenting lactose, originated from the parent non-lactose-fermenting strain through selection of a spontaneously-occurring variant which was always present in the original culture in the ratio $1 : 1 \times 10^5$. Suppose that in a bacterial culture, spontaneous variants occur whose resistance to a particular drug is higher than that of the average cells. When a heavy inoculum of this culture is added to a culture medium containing the drug, conditions would be such that the variants would outgrow the less-resistant parent cells and a resistant strain would be selected. Recent evidence indicates that some drug-resistant strains may arise in this way.

A method for distinguishing between spontaneous and induced resistance was developed by Luria and Delbrück (1943) in a study of the origin of bacteriophage-resistant strains of *E. coli*. A bacteriophage is a self-reproducing submicroscopic unit, capable of reproduction only in the presence of suitable bacterial cells ; in other words it is a virus which infects bacteria. When a pure bacterial culture is contaminated by such a virus, the culture will clear after a few

hours owing to destruction of the virus-sensitive bacterial cells. After further incubation for some hours, or possibly days, the culture may become turbid again owing to growth from a bacterial variant which has been able to resist the virus. The variant can be isolated and freed from the virus, and in most cases retains its resistance for many generations. Luria and Delbrück made a theoretical analysis of the probability distributions of the number of resistant bacteria to be expected in the two cases where the resistance developed spontaneously through a mutation, and where it developed through interaction of virus and bacteria. The observed distribution corresponded with the theoretical based on the assumption of spontaneous occurrence of resistant forms.

Demerec (1945 *a* and *b*) has applied the method of Luria and Delbrück in an analysis of the development of penicillin-resistant strains of *Staph. aureus*. The method of analysis is as follows: "If resistance is induced through interaction between bacteria and penicillin when they are in contact with each other, it would be expected that approximately similar numbers of resistant bacteria would be obtained when samples containing similar numbers of bacteria are plated in nutrient agar containing a certain concentration of penicillin, irrespective of the origin of these samples. The situation would be quite different in the event that the origin of resistance is mutational. In such case, one would expect to obtain similar numbers of resistant colonies only in samples taken from the same culture. If, however, each of the samples comes from a separate culture, and mutations occur at random, then one would expect to obtain a large number of resistant colonies from cultures in which mutation happened to occur early in the growth of the culture and a small number of resistant colonies from cultures in which mutation happened to occur late, assuming that resistant bacteria grow more or less like the normal. If resistance originates by mutation, then the variation in number of resistant bacteria between samples taken from separate cultures should be much greater than between samples taken from the same culture.

"One of the experiments to test these two possibilities

was conducted as follows :—From the same broth dilution, containing about 300 bacteria per c.c., 30 tubes were prepared with 0·3 c.c. of material each, and one tube with about 15 c.c. of the material. At the same time, 20 samples of 0·3 c.c. each from the same dilution were plated in the medium containing 0·064 units per c.c. of penicillin, to determine if any of the samples contained resistant bacteria. None was observed; and therefore it was reasonable to assume that each culture was started with an inoculum consisting of susceptible bacteria only. Cultures were incubated at 37° C. for about eighteen hours, and during that time the number of bacteria increased to about 2×10^8 per c.c.; that is, in the 30 small cultures, from about 100 to about $6{\cdot}6 \times 10^7$. The entire contents of each of the 30 tubes were plated in a Petri dish with 0·064 units of penicillin per c.c. of culture medium; and 20 samples of 0·3 c.c. each were taken from the large tubes and were similarly plated with the medium containing 0·064 units of penicillin per c.c. In each of these 50 platings about $6{\cdot}6 \times 10^7$ bacteria were placed in medium containing an identical concentration of penicillin; therefore if resistance develops through interaction between bacteria and penicillin, one would expect to find on each plate a similar number of resistant colonies. However, if resistance originates through mutation, then one would expect that the 20 samples taken from the same culture would give similar numbers of resistant colonies, while an appreciable degree of variation in number of resistant colonies would be expected among samples taken from the different cultures.

"The results show very slight variation in number of resistant colonies among the 20 samples taken from one culture. The extreme variants are 16 and 38; the average number of colonies per culture is 28·9; the variance is 39·8, X^2 is 22·7, and P is 0·3. On the other hand, the number of resistant colonies per sample taken from independent cultures varies greatly. The extreme variants are 9 and 839, the average is 120, the variance is 42,718, X^2 is 10,670, and the probability that such a distribution may be due to sampling is extremely small" (Demerec, 1945*b*).

The results of this experiment favour the assumption that resistance to penicillin originates spontaneously, and that resistant bacteria may be found in any large population. How far are we justified in terming such a change a *mutation*? There is little evidence for sexual reproduction in bacteria, and no clear distinction between nucleus and cytoplasm, but, nevertheless, recent experiments by Tatum and co-workers have shown that changes very similar to genetic mutations can be induced by exposure of bacteria to X-rays.

When cultures of *Escherichia coli* and of *Acetobacter melanogenum* were exposed to a dose of X-rays which killed 99·99 per cent. of the cells, single well-defined colonies could be isolated by plating out in complete media the irradiated suspensions, after incubation to increase the probability of obtaining cultures from the surviving cells. The strain from each colony was subcultured and tested for growth requirements in synthetic media. From 800 isolations, two strains of *Escherichia coli* were isolated, one of which required biotin and the other threonine. Four new strains of *Acetobacter melanogenum* were isolated by a similar procedure; strain (1) required glycine or serine; strain (2) adenine or adenosine; strain (3) glycine; and strain (4) probably leucine. Following a second X-ray treatment of the two abnormal strains of *E. coli*, sixteen further new strains with additional growth requirements were obtained from 1741 single colony isolations. The irradiation had significantly raised the rate at which new strains arose from 1 in 2000 for the non-irradiated strain to 16 in 2000 for the irradiated (Gray and Tatum, 1944; Tatum, 1945*a*). Cultures of the new strains were indistinguishable microscopically from the parent strain. The production by further X-ray treatment of new strains from the two induced variants of *E. coli* offers indirect support for the existence of a gene-like mechanism controlling hereditary variation in bacteria.

X-ray treatment of the mould *Neurospora crassa* is known to induce true gene mutation which is associated with certain biochemical deficiencies. Such deficiencies have been shown to result from an inability to carry out definite chemical

reactions necessary for the biosynthesis of essential metabolites from simpler nutrients. These defects are characteristic of the strain and are transmitted in accordance with Mendelian requirements. A few of the genes involved have been located in one or other of the seven chromosomes of *Neurospora*. The genes controlling biosynthesis of essential metabolites in *Neurospora* seem to act through their primary effect in determining the production of enzymes involved in biosynthesis. One enzyme effects only one step in a synthesis, and its production is controlled by one gene, so that single-mutants are characterised by their inability to synthesise a single metabolite which, before growth can proceed, must be added to the medium. A double-mutant, obtained by irradiation of a strain originating by a single mutation, develops a second deficiency in addition to its original deficiency. Thus, each deficiency acts as a biochemical marker by which a given mutant can be traced through subsequent generations (Beadle, 1945; Tatum and Beadle, 1945).

In the case of *Escherichia coli*, the sixteen strains obtained by further irradiation of the two original mutant strains each required a single growth factor *in addition to* that required by the parent strain. The double requirement of each new strain thus provides reasonable certainty of derivation of the sixteen mutants from their parent strains, and also supports the view that inheritance of biochemical character in bacteria can follow closely the pattern observed in other micro-organisms in which such changes can be related to genetic mutation. Table 21 lists the nutritive peculiarities of the double mutant strains of *E. coli*. By similar methods triple mutants were obtained which were nutritionally stable; one mutant strain required threonine, leucine and thiamin; another required biotin, phenylalanine and cystine. These two strains bred true except for an occasional variant which had reverted for a single growth factor. When a mixed inoculum of the two strains was used, a variety of progeny was produced including the original wild type *E. coli* for which no growth factors were required. These results indicate that gene recombination

had taken place by cell fusion (Lederberg and Tatum, 1946; Tatum and Lederberg, 1947).

TABLE 21

X-ray "mutant" strains of E. coli

Nutritional requirement of parent strain (single mutant)	Additional requirement of mutant strain (double mutant)	Concentration in 10 c.c. giving half maximal growth (seventy-two hours' growth)	Related substances tested	
			Active	Inactive
Biotin	Methionine	0·01 mg.	Homocystine	Na_2S, cystine
Biotin	Phenyl-alanine	0·016 mg.	...	Tyrosine
Biotin	Cystine	0·16 mg.	...	Na_2S; methionine ± serine; homocystine ± serine
Biotin	Isoleucine	0·04 mg.	Hydroxy and keto acid analogues	...
Biotin	Thiamin	0·004 μg.	...	Thiazole + pyrimidine
Biotin	Thiamin	0·003 μg.	Thiazole	Pyrimidine
Biotin	Histidine	0·003 mg.	...	...
Thiamin	Proline	0·02 mg.	...	Ornithine, arginine, glutamic acid, hydroxyproline
Thiamin	Proline	0·03 mg.	...	Ornithine, arginine, glutamic acid, hydroxyproline
Thiamin	Glutamic acid or proline	0·05 mg.	...	Ornithine, arginine, hydroxyproline
Thiamin	Leucine	0·03 mg.	Hydroxy acid analogue	...
Biotin	Yeast extract	...	...	Vitamins, hydrolysed casein
Thiamin	Yeast extract	...	...	Vitamins, hydrolysed casin
		Spontaneous Mutant		
Biotin	Proline	0·03 mg.	...	Ornithine, arginine, glutamic acid, hydroxyproline

From Tatum (1945*a*).

Further evidence for the mutation-like character of certain bacterial variations arises from a closer analysis of resistance to bacteriophage. A number of distinct strains of *E. coli*

bacteriophage are known, and acquisition of resistance to one strain does not imply resistance to all other strains. A variant may display resistance to any one virus strain, to any two, or to more than two. A variant resistant to two virus strains can be produced by subjecting a variant already resistant to one virus to the action of a second virus. The resultant doubly-resistant strain can be rendered triply-resistant by the action of a third bacteriophage; each type of resistance once acquired is transmitted to all daughter cells. The rate of mutation of *E. coli* to any one resistant form is characteristic for the bacteriophage towards which resistance is developed, and the rate of mutation to a variant which is resistant to any one virus, X, is the same, whether or not the parent strain already possessed multiple resistance to other viruses Y or Z (Anderson, 1944; Demerec and Fano, 1945).

The situation may best be summed up by quoting Demerec and Fano, who say, in discussing whether we are justified in referring to these changes as mutations and particularly as gene mutations: "Since these changes are retained by the entire offspring of the modified colonies, and the properties of the changed strain are as stable as those of the original one, we are dealing with hereditary changes. Since these changes arise, as far as we can tell, in a single discrete step, it seems legitimate and proper to call them mutations. The justification for calling them gene mutations is much less convincing. The existence of genes in higher organisms is inferred from the Mendelian segregations in crosses between unlike strains or races; the unitary nature of a gene is defined by its behaviour as a discrete unit in heredity, crossing over, mutation, and finally in chromosome breakage. Strictly speaking, it is the concurrence of all these criteria that permits us to retain the idea of discrete genes notwithstanding the evidence of their partial mutual dependence (position effects). Since bacterial reproduction, as far as is known, is exclusively asexual, inheritance in bacteria cannot be studied with the aid of the methods employed in work on higher organisms, nor can the criteria characterising gene mutations be applied

directly. We have given evidence, however, showing that several distinct types of mutational change arise in *E. coli*, each with its characteristic frequency and—what is perhaps more important in this connection—independently from one another. This is at least indicative of the existence in these bacteria of several independent self-reproducing and separately changeable entities which to that extent remind one of genes as the latter are known in sexually reproducing organisms."

[Perhaps we should add that since the above quotation was chosen and sent to press as a summary of the present position, the balance of evidence has shifted to favour sexual fusion and a gene mechanism for control of heredity in one type of bacterium, *Escherichia coli* (Lederberg and Tatum, 1946; Tatum and Lederberg, 1947; Lederberg, 1947; *cf.* Malmgren and Hedén, 1947)].

The question naturally arises, can all cases of drug resistance be attributed to selection of spontaneous mutants? Resistance to one group of drugs can often be superimposed upon resistance to another, so that any strain resistant to any one drug A, after exposure to drug B, shows double resistance to drugs A and B. Such a strain is indistinguishable from one derived in the opposite way by exposure of a B-resistant strain to drug A. Such effects are reminiscent of the "mutant" types of resistance to bacteriophage. Spontaneous variations in the enzymic capacities of strains have also been recognised. The gradual and progressive development of resistance, and the progressive loss of resistance occasionally occurring in the absence of drug, can be fitted to the mutation theory, but this does not necessarily support the theory. It can be postulated that a number of genes affect resistance to any one drug, and that a single mutation confers a limited resistance. A single mutant can undergo further mutation, giving a double-mutant which possesses a higher degree of resistance than the single-mutant, the effect of the two mutations for resistance being cumulative. A third and a fourth mutation in the progeny of the double-mutant then gives rise to highly-resistant strains. Just as mutants to more resistant forms occur, so back-mutations to less resistant

individuals could take place, and the permanence of resistance in absence of drug would then depend upon whether the back mutants could or could not outgrow the fully-resistant strain. Although the mutation theory can thus be stretched to cover all types of drug resistance, the degree of stretching required is in some cases so great that the fabric becomes suspiciously thin.

Variation in response to environment

Just because the mutation theory has been found adequate in some cases, it does not follow that it can be applied in every

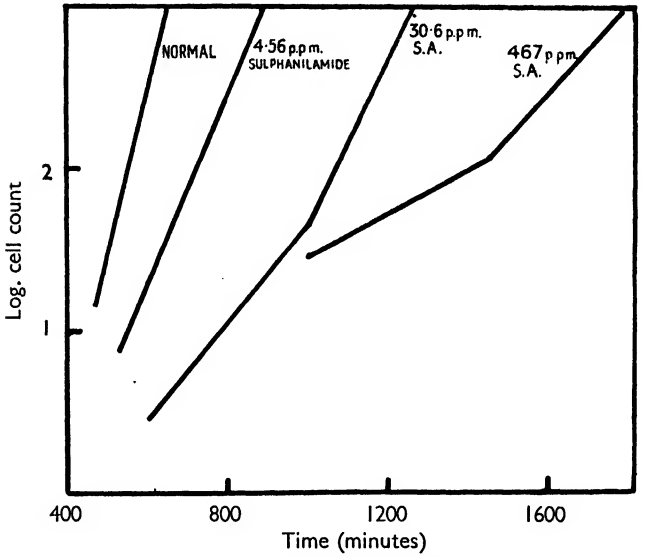


FIG. 27.—Typical growth curves for *Bact. lactis aerogenes* in presence of increasing concentrations of sulphanilamide. (Davies and Hinshelwood, 1943.)

case. Each example of drug resistance will probably have to be investigated as an individual problem, and a Lamaarkian mechanism for the development of resistance must be allowed equal status, unless resistant variants can be shown to occur spontaneously in the absence of drug with the same frequency as in its presence.

We have already seen how the enzymic balance of the bacterial cell may alter in response to changed environment,

even in the absence of cell division. Since this is so, there seems to be no serious objection to the assumption that the enzymic balance of cells of growing cultures may also alter in response to the presence of a drug in such a way that the growth-inhibitory effects of the drug are neutralised.

Davies and Hinshelwood (1943) found that sulphonamides inhibit *Bact. lactis aerogenes* not only by decreasing the rate of reproduction in a culture, but also by increasing the length of the lag phase. Using increasing concentrations of sulphonamides, plotting growth in the usual way (logarithm of cell count against time), growth curves were obtained, as indicated in Fig. 27, with greatly altered slope. During the early logarithmic phase the slope of the curve was much decreased,

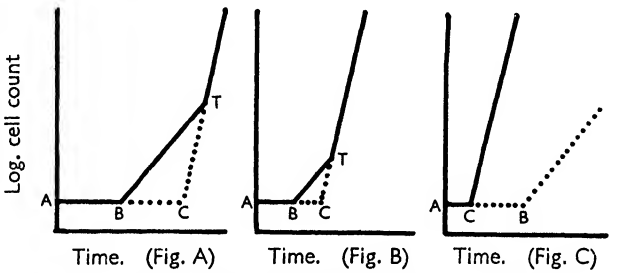


FIG. 28.—Effect of training *Bact. lactis aerogenes* to sulphonamide; shortened lag of an alternative growth mechanism. (Davies and Hinshelwood, 1943.)

but half-way through this phase a more rapid form of growth occurred, as indicated by change in slope.

These results were believed to show that two types of metabolic reaction (type I and type II) were involved in growth and in development of resistance to sulphonamides. Type II growth was slower than type I and had a longer lag period. It was not normally utilised in the absence of drugs. Type I growth was sulphonamide-sensitive, however, so that, in the presence of sulphonamides, type II growth, which was less sensitive, was called into play. The result was a superposition of type I with a shorter lag but, in the presence of sulphonamide, a slower rate, on type II which had a longer lag but a more rapid rate. The effect of this superposition is indicated diagrammatically in Fig. 28, where

the transition point from type I to type II growth is indicated by the intersection of the two growth curves. A single subculture in the presence of the drug resulted in a shortening of the lag of type II growth as shown in Fig. 28 B, and on further subculture type II lag could be shortened until type II growth completely replaced type I as in Fig. 28 C.

Repeated subculture in a sulphonamide medium reduced lag of type II growth to a limiting value. In the early stages, adaptation was reversible and resistance easily lost on subculture in the absence of drug. In later stages, when type II growth had speeded up until equal to uninhibited type I growth, drug resistance became a more stable feature of the strain. The results were interpreted mathematically to show that, during training, *Bact. lactis aerogenes* develops sulphanilamide resistance in the early stages by a shortened lag phase of a reserve growth mechanism, but that this is a temporary expedient which serves while the cells develop enzyme systems necessary for the synthesis of appropriate sulphonamide antagonists. The method of approach used by Hinshelwood, while suggestive of a complex series of interrelated processes involved in the development of drug resistance, does not provide the necessary experimental proof for the theories which it suggests.

A similar type of investigation was [illegible] proflavine, methylene blue and crystal violet (Davies, Hinshelwood and Pryce, 1944, 1945). *Bact. lactis aerogenes* reacted in essentially the same way to these three dyes and the method of development of drug resistance seemed to be similar in each case. All three drugs increased the lag phase of unadapted organisms, particularly the "early lag" of young cultures, decreased the rate of logarithmic growth and decreased the value of the stationary population. The effect on the lag phase could be completely prevented by the addition of a cell-free filtrate from a normal culture. Apparently, the three dyes increased the lag phase by inhibiting production of water-soluble essential growth-promoting substances. The process of training a resistant strain involved, among other effects, the shortening of the lag phase in the presence of

drug. Cell-free filtrates from a resistant strain had the same growth-promoting effect as filtrates from a normal culture, so presumably the resistant strain developed the ability to synthesise its own growth factors even in the presence of drug. Training also produced a rise in the rate of logarithmic growth, but again this was a slower process than the restoration of lag to normal.

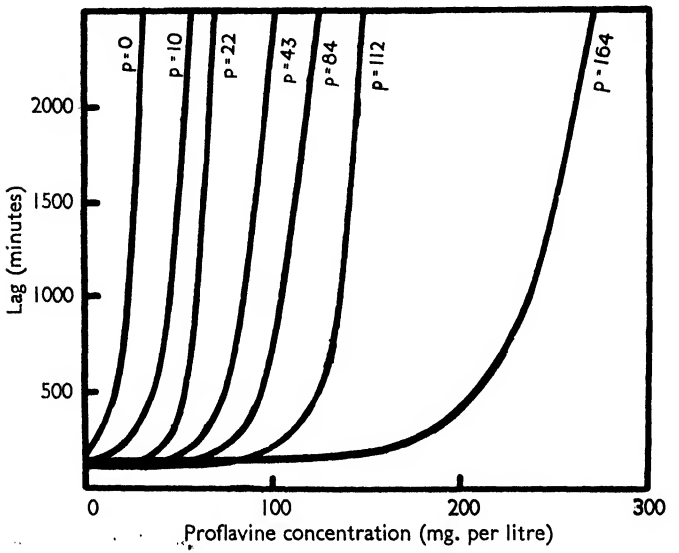


FIG. 29.—Relation between "lag" and proflavine concentration for *Bact. lactis aerogenes* trained at various concentrations (p) of proflavine. (Davies, Hinshelwood and Pryce, 1945.)

In an investigation of the degree of training of *B. lactis aerogenes* to proflavine, as measured by the decrease in lag which occurred during different stages in the growth of a culture, resistance was found to have developed by the end of three cell divisions (Davies, Hinshelwood and Pryce, 1944). This result is scarcely expected on a mutation hypothesis. It is also difficult to avoid the conclusion that there is a shift in the enzymic balance of cells in response to the presence of proflavine when the effects of increasing concentration of drug are examined. During training of *B. lactis aerogenes* to resist proflavine, the degree of adaptation is exactly graded to

correspond to the concentration of drug in which the organism is grown (see Fig. 29). The same organism adapted to low concentrations of sulphanilamide becomes resistant to high concentrations (Hinshelwood, 1946).

It is quite possible to explain most of Hinshelwood's results on the basis of selection of spontaneous mutants, but several of the observed effects can be more satisfactorily explained without resort to such a hypothesis. There is general agreement that partial resistance is much less stable than complete resistance, which behaves as an inherited character Partial resistance often develops very rapidly, and has much in common with adaptation of bacteria to new metabolites. Alteration in enzymic balance in response to changed environment may take place without acquisition by a cell of any new enzymes. Reduced lag after subculture in low concentrations of drug could be achieved simply by increased intracellular accumulation of an essential metabolite which, in absence of drug, was utilised completely as fast as it was formed. Adaptation of this type would not be expected to possess the stability resulting from selection of mutants.

Selection of induced variants

Two possible general mechanisms for development of resistance have been considered—selection of occasional spontaneous mutants, and specific modification of enzyme balance in all cells of a homogeneous population. A third possibility which must be admitted is induction of mutation and simultaneous selection of suitable mutants by the same drug.

The work of Avery, MacLeod and McCarty (1944) leaves no doubt that application of an organic compound to a growing culture can alter the morphological and serological characters and synthetic capacity of a bacterial population in a predictable and reproducible manner. When nucleic acid, isolated from heat-killed type III pneumococcus, was added to a culture of non-encapsulated non-virulent pneumococcus (from type II), a transformation of type took place. Type II organisms were transformed into type III organisms indistinguishable both serologically and morphologically from the

type III strain from which the nucleic acid was isolated, and also capable of providing fresh supplies of nucleic acid for transformation of another culture. There is no doubt that the nucleic acid acted upon the culture from type II by altering its enzymic make-up so that it developed the capacity to synthesise the specific polysaccharide which forms the capsule of type III. The induced variation was not a random change but was predictable, always corresponding in type-specificity to that of the encapsulated cells from which the transforming substance was isolated. Similar effects have been studied by Boivin, Vendrely and Lehoult (1945) in *Escherichia coli*, and by Weil and Binder (1947) in *Shigella paradysenteriæ*. These results suggest many interesting possibilities in the field of bacterial inheritance which are scarcely relevant to the problem of drug resistance, but they also provide an example of the controlled induction of a change which closely resembles mutation by contact of a growing culture with a foreign substance.

We have already cited in detail the evidence that penicillin resistance in *Staph. aureus* develops by selection of spontaneous mutants. Similar conclusions have been reached in an analysis of sulphonamide resistance in *Staph. aureus* (Oakberg and Luria, 1946). The *in-vitro* development of streptomycin resistance by *Shigella dysenteriæ* has also been explained as due to the selection of spontaneous mutants, but, as emphasised already, each case of resistance must be analysed as an individual problem, and no general conclusion can be reached from a study of a few cases (Klein and Kimmelman, 1946). As already mentioned (p. 272) resistance to streptomycin may develop by two distinct mechanisms, even in a single strain of meningococcus. All these drugs are usually regarded as purely selective in their action. It is possible that some of them exercise, in addition, an inductive effect.

It is apparent from the cases cited above that mutation or some closely allied process can be induced in a controllable manner by suitable biological extracts. It might be argued that this was a special case and that mutation could not be induced, far less controlled, by simple chemicals. This view was valid until quite recently, but must now be abandoned in

the face of accumulating evidence to the contrary. Induction by allylisothiocyanate or by "mustard gas" to increased random mutation in higher organisms has been conclusively demonstrated (Auerbach, Robson and Carr, 1947). The carcinogenic hydrocarbon 1 : 2 : 5 : 6-dibenzanthracene has also been found to induce random mutation in *Drosophila* (Demerec, 1947). There have been suggestions that sulphonamides may influence chromosomal arrangement in plants and in *Drosophila*, but up to the present these reports have not been confirmed (Beal, 1942; Thomas and Chevais, 1943).

Ark (1946) has claimed that acenaphthene induced mutation in *Phytomonas michiganensis* and in *Erwinia carotovora*, but the basis for his distinction between induction and selection is not clear; in plants where full genetic analysis is possible, acenaphthene and colchicine are both admitted to have a direct effect on nuclear division. A preliminary study of sulphonamide resistance in *Neurospora* has been made by Emerson and Cushing (1946). In this organism full genetic analysis is possible, and the conclusion was reached that partial adaptation did not involve gene mutation, but that the fully-adapted strain was a true mutant. Despite the advantage of handling an organism in which genetic change could be analysed, it was not possible to distinguish between a "selection" and an "induction" mechanism. Exposure of *Neurospora* to "mustards" results in increased random mutation. The mutants have been characterised by their growth factor deficiencies (Horowitz, Houlahan, Hungate and Wright, 1946). Di-β-chlorethylamine, a "nitrogen mustard," has been found effective for the production of biochemically-altered strains in bacteria as well as in *Neurospora*. Many of these strains show specific growth factor requirements which correspond exactly with deficiencies known to be related to genetic mutation in *Neurospora* (Tatum, 1946). There is thus no barrier to the conclusion that bacterial mutation can be induced by simple chemicals. Although such "mustard"-induced mutation as was studied in detail was random in nature, there is no *a priori* reason to assume that chemicals will only exercise a random effect. A study by Carr (1947)

of the induction of mutation in mice by 1 : 2 : 5 : 6-dibenzanthracene suggests that one locus in a chromosome may be more susceptible to mutation induced by dibenzanthracene than any other, so that each member of a population of cells exposed to the chemical is liable to undergo the same mutation.

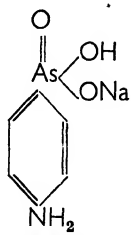
We are left with the conclusion that, up to the present, there is no evidence which excludes the possibility of drug resistance developing by any of the mechanisms we have discussed.

Drug resistance in trypanosomes

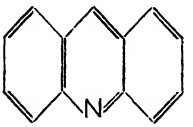
The study of drug resistance in trypanosomes developed quite separately and along different lines from investigation of bacterial resistance. The distinctive path of research may be ascribed partly to historical chance in that chemotherapy of trypanosomiasis developed long before bacterial chemotherapy, and partly to technical limitation in that it was not possible to maintain pathogenic trypanosomes in an undegenerate form *in vitro*, and all early studies had to be made on animals.

It will be remembered that a resistant strain of *Trypanosoma brucei* was first developed in Ehrlich's laboratory by the parafuchsin treatment of infected mice (Chapter I). The initial treatment caused parasites to disappear from the peripheral blood, but after a week or two they reappeared. When this cycle of events had been repeated several times on an infected animal, the trypanosomes developed resistance to parafuchsin. These results were soon extended, and strains resistant to a variety of dyestuffs and to organic arsenicals were produced. Trypanosomes made resistant to atoxyl were also resistant to other phenylarsonic acids, and to a group of dyes built on a common structural pattern, the acridine, oxazine, thiazine and selenazine dyes; but not to dyes of the triphenylmethane or trypan blue group. Ehrlich and his colleagues later developed strains resistant to trypan blue and trypan red and concluded that drug-resistant trypanosomes could be divided into three groups, arsenic resistant, basic triphenylmethane dye resistant and azo dye resistant. Parasites resistant to any one dye of the triphenyl-

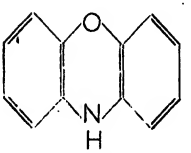
methane group were also resistant to all other dyes of this group, but not to azo dyes or to atoxyl; in the same way



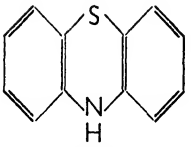
Atoxyl



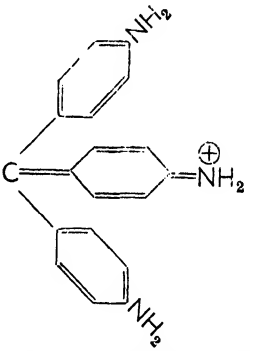
Acridine



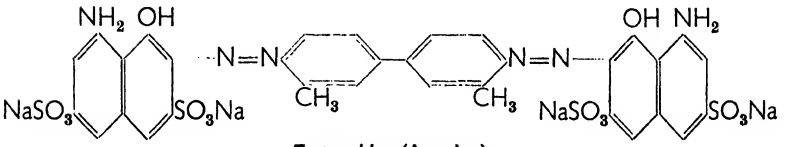
Oxazine



Thiazine



Parafuchsin (Triphenylmethane dye)



Trypan blue (Azo dye)

trypan blue-resistant strains possessed group resistance to all related azo dyes, but not to triphenylmethane dyes or to organic arsenicals. It was soon noticed that strains rendered resistant to a dye were not stained by contact with that dye, whereas susceptible parasites were readily stained. Much of the subsequent research into drug resistance in trypanosomes has been concentrated on efforts to establish the cause of this difference.

Ehrlich believed that his chemoreceptor theory could be adapted to explain these phenomena. He suggested that there

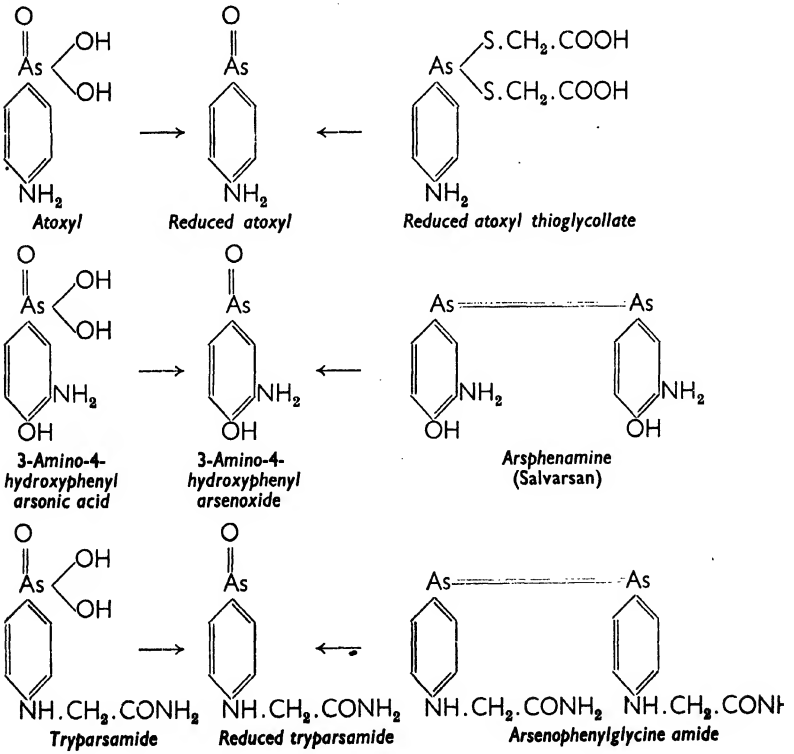
existed in the cell specific groups or chemoreceptors which combined with certain groups in the drug molecule. Drug resistance was regarded as due to an alteration in the affinity of the receptor for the drug to which the strain had been made resistant. In 1924 Voegtlin, Dyer and Miller suggested that two other explanations of drug resistance should be considered; firstly, a changed permeability of the cell membrane, and secondly, a physico-chemical defence mechanism of the protoplasm against the toxic action of a drug, possibly by production of a chemical which could neutralise the effects of the drug. Voegtlin had already shown that trypanosomes could be protected against arsenoxides by sulphydryl compounds. He now suggested that variation in the arsenic resistance of different strains depended upon the amount of "SH antagonist" produced by the cell, in excess of its basal requirements.

As is so often the case, further progress was limited by technical difficulties; pathogenic trypanosomes such as *T. brucei* or *T. rhodesiense* which were killed by organic arsenicals could not be subcultured *in vitro* without undergoing change to a degenerate form. *T. cruzi*, which could be subcultured *in vitro*, was not susceptible to arsenicals. Following upon the publication in 1930 by Yorke and Murgatroyd of a method of keeping trypanosomes normal at 37° for 24 hours, it was possible to submit the theories on the mechanism of drug resistance to experimental test. Strains made resistant to reduced tryparsamide were found to remove little of that drug from the culture fluid, while normal trypanosomes removed large amounts of the drug (Yorke and Murgatroyd, 1930; Yorke, Murgatroyd and Hawking, 1931). Voegtlin's first theory, altered permeability, would therefore appear more probable than his second. If resistant trypanosomes produced "SH antagonists" in greater abundance than normal trypanosomes, the resistant strains would be expected to fix larger quantities of organic arsenicals than non-resistant strains.

The failure of resistant trypanosomes to remove tryparsamide from the medium might however be attributed, not to a decreased permeability of the cell membrane, but to a

decreased affinity of the cell protoplasm for the drug. In other words, as suggested by Ehrlich, the " chemoreceptors " of the resistant strain could be supposed to have a lowered affinity for the drug. Much evidence has now accumulated which indicates that such an explanation is unlikely, at any rate as far as organic arsenicals are concerned (Hawking, 1937; King and Strangeways, 1942; Eagle and Magnuson 1944).

Pentavalent arsenicals such as atoxyl, arsacetin and tryparsamide are known to be inactive as such, but are probably reduced *in vivo* to the trivalent arsenoxide form. The arseno drugs such as arsphenamine and the thioarsenites are also believed to be toxic only in the arsenoxide form, and in practice trypanosomes made resistant to pentavalent arsenicals, thioarsenites or arseno drugs have always been found resistant to the corresponding arsenoxides.



Arsenoxides are probably toxic because they form stable thioarsenites with the essential sulphydryl groups of enzymes (Chapter IV). If we supposed that drug-resistant trypanosomes had acquired a lowered affinity for arsenoxides, it would be necessary to conclude that in resistant forms all the "SH enzymes" were either dispensed with or altered into some form where the sulphydryl group was no longer essential for enzyme activity. In view of the multiplicity of "SH

TABLE 22

Relation between drug resistance and drug adsorption in normal and tryparsamide-resistant trypanosomes

Drug	Approximate percentage of available drug adsorbed by		Ratio: Adsorption Normal / Adsorption Resistant	Ratio: Trypanocidal concentration for resistant strain / Trypanocidal concentration for normal strain
	Normal Trypanosomes	Resistant Trypanosomes		
Reduced tryparsamide	Nearly 100	?5	20	265-512
Halarsol	70-90	?5-10	10-20	32
Phenylarsenoxide	70-100	70-100	1	1
Arsenophenylglycine	25-50	20-40	1	2
Sodium arsenite	40	40	1	1
Tartar emetic	50	50-75	1	1

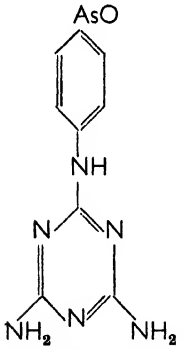
From Hawking (1937).

enzymes" such a mechanism for the development of arsenoxide resistance seems improbable. This conclusion is reinforced by observations that the specificity of resistance depends, not on the arsenoxide radical, but on the nature of the organic residue to which it is attached. Atoxyl-resistant strains show equal resistance to tryparsamide, but are only slightly resistant to arsenophenylglycine or sodium arsenite; moreover, atoxyl-fast strains adsorb sodium arsenite and arsenophenylglycine to the same extent as normal strains (see Table 22). Living

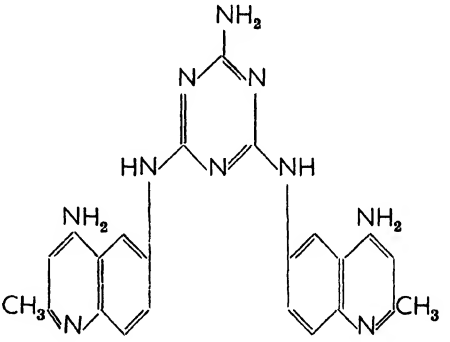
trypanosomes take up little of an arsenical to which they are resistant, but there is a rapid intake and fixation as soon as the culture is killed (Yorke and Murgatroyd, 1930; Yorke, Murgatroyd and Hawking, 1931, 1932; Yorke, 1932; Hawking, 1937).

Full discussion of the structure and permeability of cells has been postponed until Chapter VII, but it is necessary here to draw attention to the existence of organisation within micro-organisms. As we have said, it is impossible to attribute tryparsamide resistance to a loss of "SH-enzymes"; the tryparsamide-resistant cell must, therefore, protect its "SH-enzymes" from contact with drug. We are apt to regard a cell as a bag of enzymes contained within an inert semi-permeable membrane, but there is considerable evidence for organisation within the membrane and for the existence of an active transport mechanism for the uptake or excretion of certain foods. An organism might forestall the ultimate lethal action of a drug either by elimination of its transport across the cell wall or by metabolic conversion of the drug, within the cell wall, to a chemical form close to that of a normal excretory product. Both these hypotheses imply the possibility of dual character within a drug, a character governing uptake, and a character governing lethal action within the cell. These concepts can be profitably applied to a study of drug resistance in trypanosomes. The term "arsenic resistant" as applied to trypanosomes is a misnomer, since the resistant strains are not actually resistant to arsenic but only to those arsenical drugs belonging to one particular chemical type; thus, trypanosomes made resistant to aromatic arsenicals such as atoxyl are cross-resistant to many other aromatic arsenicals but are not cross-resistant to various carboxyphenylarsenoxides (King, 1943), or to γ-(*p*-arsenophenyl)-butyric acid (Eagle, 1945*b*). The suggestion was made by King that although the final chemical action which results in death of the trypanosome was the same for all arsenic derivatives, each chemical type entered the trypanosome by a different mechanism. Recent evidence suggests that the carboxyl-substituted aromatic arsenicals may indeed

be involved in a metabolic reaction prior to their ultimate lethal combination with SH groups. *p*-Aminobenzoic acid interferes with the trypanocidal action of arsenophenylbutyric acid but not with that of 3-amino-4-hydroxyphenylarsenoxide (mapharsen), while glutathione interferes with the action of both. This evidence has been extended by Williamson and Lourie (1948) in a study of melarsen oxide (formula below), a representative of a further class of arsenoxides active against tryparsamide-resistant trypanosomes. The type of compound in which to seek for selective interference with " uptake " of melarsen oxide would be a substance containing the melamine nucleus. It was in fact found that bis(2-methyl-4-amino-6-quinolyl)-melamine (surfen C) interferes selectively with melarsen oxide, whereas glutathione interferes equally with the toxic action of melarsen oxide and other arsenoxides.



Melarsen oxide



Surfen C

A similar distinction between mechanism of uptake and mechanism of lethal action seems to exist with the antimalarial drug paludrine. Paludrine resistance is apparently directed against uptake rather than against lethal action since paludrine-resistant *Plasmodium gallinaceum* is not resistant to pyrimidine precursors of paludrine (Williamson and Lourie, 1947). Schueler (1947) suggests that resistance may be achieved by a shift in the isoelectric point of a protein which normally binds the drug.

Arsenical resistance has been subjected to much fuller analysis than other types of drug resistance in trypanosomes,

but strains have been developed resistant to antimony compounds, and to the more recently discovered groups of trypanocidal drugs, such as styryl quinolines, Bayer 205, the guanidines and the amidines. Cross resistance between the arsenicals, Bayer 205 and the guanidines or amidines is unknown, so that the mechanism of resistance to each group of drugs may be quite different. The development of resistance may involve altered permeability in some of these cases also (Jancsó, 1931).

We must again emphasise that the term " drug resistance " is only a relative one. In no case has a strain been developed which was completely resistant to a drug *in vitro* but in many cases sensitivity is decreased several hundred-fold, so that a resistant strain would not be cleared from an animal by the maximum tolerated dose of trypanocidal drug.

Origin of resistant forms of trypanosomes

The capacity for resistance to a drug, once acquired, is usually a fairly permanent character of the strain. Atoxyl-resistant strains of *Trypanosoma rhodesiense* were maintained by Yorke for twelve and a half years, during which the strain passed unchanged through a series of 1500 mice (Fulton and Yorke, 1941*a*). Transmission of tryparsamide-resistant *Trypanosoma brucei* four times through the insect vector *Glossina morsitans* was also achieved without loss in resistance (Yorke, Murgatroyd and Hawking, 1933; Murgatroyd and Yorke, 1937). Resistance to other drugs is not usually as permanent as atoxyl resistance; thus, amidine and guanidine resistance declined in less than one year, and all trace of resistance had disappeared after three and a half years (Fulton and Yorke, 1941*a*). Resistance to Bayer 205 also tended to decline, but a particularly resistant strain maintained its resistance unimpaired for three and a quarter years. Drug resistance therefore behaves as an inherited character through many generations.

Resistance is usually developed *in vivo* in mice by daily administration of such doses of drug as just fail to clear the peripheral blood of parasites. By subjecting trypanosome

suspensions to a series of short exposures to the highest concentration of drug which just fails to kill all the parasites, resistant strains have been developed *in vitro* indistinguishable from those produced by the mouse method. The rate of development of resistance *in vivo* varies greatly with different drugs ; thus Yorke found that resistance to atoxyl developed in four to eight weeks, halarsol resistance developed in three months, while twelve months' treatment was necessary to produce resistance to Bayer 205. The difficulty of developing resistance to Bayer 205 may be related to the fact that the reticulo-endothelial system normally takes up this drug very quickly after injection. Resistance developed much more quickly if the reticulo-endothelial system was eliminated as far as possible by splenectomy before treatment with drug and by intravenous injection of copper a few hours after (Yorke, Murgatroyd and Hawking, 1932 ; Jancsó and Jancsó, 1935*a*).

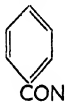
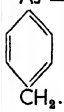
These data on development of resistance are consistent with, but do not supply evidence for, a hypothesis involving selection of mutants. Eagle and Magnuson (1944) have observed a case of spontaneous development of resistance in the absence of drug which supports a mutation theory. Their strain was resistant to amino- and amide-substituted phenylarsenoxides, such as reduced atoxyl and reduced arsacetin, but not to acid-substituted or unsubstituted phenylarsenoxide. It was thus closely similar to resistant strains arising through treatment of infections with sub-curative doses of trypanocidal drugs of the atoxyl group. The similarity was carried over to the binding of arsenicals ; the spontaneously-resistant strain, like an induced-resistant strain, bound far less aminophenylarsenoxide than the parent susceptible strain, but had an unchanged affinity for arsenoxides to which it was not resistant (Table 23).

Possibly, spontaneous variation of the type described occurs in trypanosomes more frequently than is indicated by such isolated observations. Both Browning (1931) and Morgenroth (1924) noted change in resistance in the absence of any contact with drug. When resistance is found after repeated

exposure to a specific drug, the drug itself may have nothing to do with the production of variation, but may act as a selective factor which permits spontaneous variation to become apparent by killing off the susceptible trypanosomes.

TABLE 23

Relation of drug uptake to drug resistance in a spontaneous variant of T. equiperdum

Drug	Strain	Relative Trypanocidal Activity/mole	Number Trypanosomes per c.c. × 10^6	Concentration added Arsenical μg./c.c.	Per cent. Total Arsenic bound after 1 min.	Per cent. Total Arsenic bound after 10 min.
	Parent	19·0	220-330	2·0	28·7	50·5
	Variant	0·28	200	2·0	...	0
	Parent	14·0	280	1·66	...	38
	Variant	0·64	250	1·66	...	1·8
	Parent	100·0	250-280	1·66	...	71-79
	Variant	100·0	250	1·66	...	80
	Parent	54	260-280	1·66	...	49-55
	Variant	54	250	1·66	...	59

Eagle and Magnuson (1944).

The fact that resistance, apparently induced by repeated exposure to a drug, is not necessarily specific for that drug, is in keeping with the idea that resistance is not necessarily induced, but may be a spontaneous inherited variation for which any one of several drugs has the same selective action.

Drug resistance in other micro-organisms

Although various other organisms have been trained to resist chemotherapeutic drugs, none of these has been studied with the thoroughness of bacterial or trypanosome resistance.

Strains of *Babesia canis* have been made resistant to 4 : 4′-diamidinostilbene and showed cross-resistance to 4 : 4′-diamidinodiphenoxypropane and to alcaprin, while the flagellate *Polytomella cæca* and *Entamœba histolytica* may develop resistance to sulphonamides (Lourie and Yorke, 1939 ; Fulton and Yorke, 1941*b* ; Lwoff, Nitti, Tréfouël and Hamon, 1941 ; Rodaniche and Kirsner, 1942).

Although the expression " quinine resistant " has been repeatedly used by clinicians when referring to malarial patients who appeared to react poorly to quinine therapy, there is as yet no satisfactory evidence that malaria parasites acquire an enhanced resistance to quinine following treatment of a human infection with sub-curative doses of quinine. Some slight signs of resistance have been reported following repeated passage of *Plasmodium relictum* in quinine-treated birds (siskins), the parasite eventually showing a shorter incubation period in infected birds treated with quinine than the parent strain. This resistance was sometimes, but not invariably, retained in passage through the insect vector, *Culex pipiens* (Kritschewski and Rubinstein, 1932 ; Kritschewski and Halperin, 1933). Attempts to develop a quinine-resistant strain of *Plasmodium cathemerium* in canaries were completely unsuccessful (Lourie, 1935).

A plasmoquine-resistant strain of *Plasmodium knowlesi* was secured by Fulton and Yorke (1941*c*, 1943) following passage of the parasite through a series of plasmoquine-treated monkeys. The plasmoquine resistance persisted, in part at least, during a period of five months while the strain was maintained in a monkey in a chronic state of infection as the result of treatment with a small dose of atebrin. Resistance to the new antimalarial drug paludrine develops readily in *Plasmodium gallinaceum* and persists after transmission of

the parasite through the mosquito (Bishop and Birkett, 1947 ; Williamson, Bertram and Lourie, 1947).

Clinical significance of drug resistance

Micro-organisms appear to be capable of developing resistance to the majority of drugs which are employed clinically. In view of the cross resistance shown between different groups of drugs, it is obviously important that the clinician should have information as to which groups of drugs may be usefully tried in infections with resistant strains. For example, the fact that resistance to any one *p*-aminobenzenesulphonic acid derivative means resistance to all benzenesulphonic acids possessing an aromatic *p*-amino group, implies that there is little use in changing over to a different sulphonamide in an infection which does not respond to the first sulphonamide tried. The *p*-aminomethylbenzenesulphonic acid derivatives, such as marfanil or V. 187 (Evans, Fuller and Walker, 1944), do not show cross resistance with sulphonamide-resistant strains, and could therefore be used to treat a sulphonamide-fast infection.

That drug resistance can become a serious menace to the usefulness of the sulphonamides was seen from the decline in response to sulphonamides of gonorrhœal infections which had occurred in U.S.A. before penicillin therapy came into general use. In 1940-41 about 70 per cent. of the cases of gonorrhœa treated in one American clinic were promptly cured by sulphonamide therapy, while the remaining 30 per cent. did not respond ; four years later the proportions were approximately reversed (Mueller, 1945). Fortunately, sulphonamide-resistant strains are not cross-resistant to penicillin, but the demonstration that double resistance to two groups of drugs can be developed experimentally in the absence of cross resistance between the groups, makes it quite conceivable that strains may eventually develop resistant both to penicillin and to sulphonamides (Spink, Hall and Ferris, 1945). This difficulty is particularly likely to arise from indiscriminate prophylactic use of chemotherapeutic drugs. In United States army camps introduction of sulphadiazine, prophylactically,

caused an initial drop in the incidence of respiratory infections, but this was followed by a gradual increase in group A hæmolytic streptococcal infection caused by a strain resistant to sulphadiazine (Damrosch, 1946). Although penicillin is now used on the widest possible scale there have been, as yet, no reports of a similar generalised increase in penicillin-insensitive strains of Gram-positive pathogens. The very limited state of our knowledge of the nature and stability of penicillin resistance is clearly demonstrated by the interesting report of Voureka (1948) that penicillin-resistant strains of staphylococci and streptococci can be rendered penicillin-sensitive by treatment with bacterial autolysates. In assessing this work, it should be remembered that several other groups of workers have obtained unstable penicillin-resistant bacterial strains (Spink and Ferris, 1947 ; Miller and Bohnhoff, 1947*b*). Streptomycin provides a complete contrast to penicillin ; already, after brief clinical trial, the development of streptomycin resistance is seen to be a serious limitation to its usefulness (Alexander and Leidy, 1947 ; Pyle, 1947).

The danger of development of trypanosome strains resistant to arsenicals has been emphasised by Yorke (1932) and by Lourie and Collier (1943).

Association of other changes with development of resistance

Some drug-resistant strains of bacteria have been found not to differ in morphology, antigenicity or in virulence from the normal parent strains, but a number of individual cases of alteration in form or virulence have been reported. A marked and permanent decrease in virulence was observed with a strain of pneumococcus rendered resistant to sulphathiazole, but reduction of virulence could not be directly correlated with degree of resistance (Horsfall, 1942). One strain of *Shigella paradysenteriæ* lost its virulence during development of sulphonamide resistance, another remained virulent (Cooper and Keller, 1942). Various strains of pneumococci became avirulent when made resistant to penicillin (McKee and Houck, 1943). Change in form as well as in virulence has been reported ; with some strains of

Streptococcus hæmolyticus, the virulent mucoid strain passed to a smooth avirulent strain during training to sulphonamide ; virulence was not regained by mouse passage (Hadley and Hadley, 1941). However, although change in form and change in resistance may be produced at the same time, the two phenomena are not interdependent and may be separately modified (Cutts and Troppoli, 1942).

Strains of sulphonamide-resistant staphylococci, whether prepared by *in-vivo* or *in-vitro* techniques, were found by Spink, Hall and Ferris (1945) to be equally virulent, but strains made resistant to penicillin *in vitro* lost their virulence ; penicillin-resistant strains developed *in vivo* retained their virulence. Virulence and resistance seem also to be independent variables so that, generally, a strain made resistant *in vivo* retains its virulence. A strain made resistant *in vitro* frequently loses virulence during development of resistance. Virulence can sometimes be restored to the avirulent form without loss of resistance (Schmidt, Sesler and Dettwiler, 1942).

CHAPTER VII

THE RELATION OF STRUCTURE AND ACTIVITY

In 1868, Crum Brown and Fraser found that various alkaloids, possessing the most diverse pharmacological actions, could be reacted with alkylhalides to form quaternary ammonium derivatives which possessed the property of paralysing the motor nerve endings in the same manner as curare (Brown and Fraser, 1868, 1869 *a* and *b*). Curare-like action was subsequently shown to be a general property of "onium" ions (Ing, 1936). This relationship between chemical structure and biological activity served to stimulate a vast amount of speculation and research. In 1910 Barger and Dale were able to demonstrate a number of regularities in the relation of structure to activity among pressor amines, but on the whole the results of many other similar investigations were disappointing. Even so, classical pharmacology could boast, until recently, considerably greater success in this field than could chemotherapy.

In seeking a relationship between structure and chemotherapeutic activity, chemists have tended to neglect the biological variables involved, while pharmacologists, as well as chemists, were until recently ignorant of those changes in electronic configuration which might result from an apparently small change in chemical structure. If a micro-organism is to be capable of producing anything more than a mild chronic infection, it must be able to multiply in the host tissues at such a rate that the defence mechanisms of the host (phagocytosis, antibody production, etc.) are overcome. A fine balance frequently exists between the invasive process and the defence mechanism, so that any drug capable of slowing down the rate of parasite reproduction may tip the scales in favour of the infected animal and may therefore be a potential chemotherapeutic remedy. However, the processes of phagocytosis, antibody production and tissue repair require time.

During this time the parasite reproductive rate must be kept below a certain critical level by maintenance of a suitable concentration of drug in the parasitic environment. Overall chemotherapeutic activity is thus a property related to a number of variables, and it is idle to expect to find a close relationship between structure and overall activity. A given change in structure may have one effect on uptake of drug by the host, a different effect on distribution within the host, and still another effect on the metabolic fate of the drug or on its toxicity to the animal. At the same time, the same change in structure may also alter the degree of uptake of drug by parasite, or the efficacy of the drug as a parasitic enzyme inhibitor. It is the combined result obtained by summation of these variables which is measured in the usual routine mouse test, and, in default of more detailed information regarding the effect of change in structure, many a compound of potential chemotherapeutic value may be overlooked; its burial place is the store cupboard; its obituary notice is an obscure corner of "Beilstein." The sulphonamides were interred for thirty years before exhumation. How many more of the vast number of organic compounds, natural and synthetic, which were given a brief test and forgotten may be worth more extended trial?

The ensuing analysis of the relation of structure to activity should be regarded, not as an attempt to find rules which may guide the chemist in the synthesis of new drugs, but rather as an opportunity to seek hints regarding the various factors governing biological response. For the purpose of discussion we shall divide consideration of the effect of change in structure into three sections:—

(1) Factors affecting distribution of drugs in animals.
(2) Factors affecting the *in-vitro* action of drugs and penetration of the bacterial cell wall.
(3) Factors affecting the interaction of drug with enzymes.

Drug Distribution

An infection may remain comparatively localised, spreading no further than the intestine, urinary tract, lymphatic system,

blood or central nervous system. If a drug is to be effective, its concentration at the site of infection must be high; the effect of change in structure on the distribution of the drug in the host must therefore be known. The importance of this subject was clearly recognised by Ehrlich, who opened the field with a study of the distribution of dyes.

The first consideration in distribution of a drug given by mouth is its rate of penetration of the intestinal wall. Studies of intestinal permeability to drugs have been made chiefly by the "isolated loop" technique. In this method, the body cavity of an animal is opened and a section of the intestine isolated by ligatures, while its normal blood circulation is maintained. The section is washed out and filled with a measured amount of experimental solution. Absorption is measured by analysing material remaining in the loop after various time intervals. This method has been criticised by Cori (1925) on the grounds that experimental disturbance of the intestine may upset its normal metabolism. He has developed a method of studying absorption in the intact animal. Rats were divided into groups and fed a measured amount of drug; after varying periods of time, groups of animals were slaughtered and the intestinal contents analysed.

Studies on the absorption of non-electrolytes by such methods immediately showed that two distinct types of absorption must be considered, passive and active. If absorption were dependent entirely on passive diffusion, one would expect the rate of absorption of any compound to be proportional to its concentration and to its diffusion constant. Among the monosaccharides, glucose (a physiological sugar) is absorbed from the intestine three times as rapidly as xylose (Cori, 1925); further, the absorption of glucose is not proportional to concentration (Verzar, 1935). These results indicate that glucose is absorbed by a specific metabolic mechanism. This conclusion was supported by the effect of phlorizin on glucose absorption; phlorizin was reported to be a specific poison for the phosphorylation of glucose (Lundsgaard, 1933), and in its presence the rate of glucose absorption from an isolated intestinal loop is lowered considerably (Donhoffer,

1935). More recent results show that glucose phosphorylation is not inhibited at physiologically-effective concentrations of phlorizin but that, at these low concentrations, the dehydrogenase systems which supply energy-rich phosphate bonds are inhibited both *in vitro* and *in vivo*. The observed inhibition of glucose uptake in the presence of phlorizin is thus due, not to direct inhibition of phosphorylation, but to interference with the supply of energy necessary for a process of active transfer (Shapiro, 1947). Amino acids also appear to be absorbed by an active rather than a passive process (Höber and Höber, 1937). The assumption would appear to be reasonable, therefore, that any substance which is a normal cellular metabolite, or which is sufficiently closely related chemically to a normal metabolite to be caught up in the same type of metabolic process, is likely to pass through the intestinal membrane by a process of active absorption.

The two aliphatic amides, acetamide and butyramide, both penetrate by a process of passive diffusion. When their rates of absorption from solutions of equal concentration were compared, butyramide was found to penetrate more rapidly than the much smaller molecule acetamide (Höber and Höber, 1937). The rate of free diffusion of acetamide in water is much greater than that of butyramide, so that the intestinal wall must be preferentially permeable to butyramide. This observation immediately raises the question as to why the cell membrane should show preferential permeability to the larger molecule, and takes us back to the pioneer studies of Overton and Meyer.

All cells have a definite structure. The main phase, the protoplasm, is an aqueous solution which is separated from the outside environment by the plasma membrane. Any substance passing into a cell must penetrate the plasma membrane. Overton (1901, 1902) and Meyer (1899) found that the rate of diffusion of organic non-electrolytes into the intact cell depended upon the lipoid-water distribution coefficient of the substances (Table 24). Overton concluded from his results that the plasma membrane was "lipoid" in nature.

The degree of correlation between lipoid solubility and penetrant power was in some cases quite remarkable. The existence of lipoid-insoluble substances which penetrate cells readily, and the marked difference in permeability shown by cells of different species (Davson and Danielli, 1943), has tended to obscure the straightforward picture presented by Overton and has necessitated the introduction of a pore theory as a supplement to the original lipoid theory.

In attempting to answer the question as to the nature of passive diffusion across a membrane we can postulate two types of processes. Either the membrane may be regarded as a continuous layer of water-insoluble substance, a lipoprotein skin, or it may be a solid mosaic with a sieve-like

TABLE 24

Relative rates of penetration of organic molecules as found by Overton

Rapid penetration . . .	Monohydric alcohols, aldehydes, ketones, hydrocarbons, weak organic acids and bases.
Less rapid penetration . .	Dihydric alcohols and amides of monobasic acids.
Slow penetration . . .	Glycerol, urea, thiourea.
Very slow penetration . .	Hexahydric alcohols, amino acids, etc. (*cf.* Höber, 1945).

structure. In the first case, the rate of diffusion of solute across the membrane will depend largely on the concentration gradient set up between the membrane and the interior protoplasm of the cell. The concentration gradient will in turn depend upon the distribution coefficient of solute between cell membrane material and external solvent. In the case of a sieve membrane, on the other hand, diffusion will take place through the holes which directly connect the aqueous phases on both sides of the membrane, and the rate of diffusion will depend upon the size of the pores and the molecular volume of the solute.

As already noted, butyramide (large molecular volume) penetrates the intestinal wall more rapidly than acetamide; the oil/water distribution coefficient of butyramide is greater than that of acetamide, so that one is inclined to conclude

that absorption from the intestine may be influenced by lipoid/water distribution coefficient. Other factors obviously enter, however, since the oil-soluble plant phytosterols, allocholesterol and the isomeric dihydrocholesterols are not absorbed at all from the intestine (Schönheimer and Hrdina, 1932; Schönheimer, Behring and Hummel, 1930), and, as already noted, certain oil-insoluble substances, such as glucose, pass readily.

Data on the rate of absorption of chemotherapeutic drugs from the intestine are unfortunately too scanty at present to permit of any conclusion as to the connection between rate of absorption and structure, apart from the general one that substances which are readily soluble in both lipoid and water appear to penetrate most rapidly. Travell (1940), using the isolated loop technique, studied the effect of *p*H on the rate of absorption of alkaloids in the cat, and came to the conclusion that when the reaction of the gastric juice was strongly acid there was no alkaloid absorption. The rate of absorption was closely related to the concentration of free undissociated base, so that, as the *p*H was raised, the rate of absorption increased. These results are in general agreement with those of Ellisor and Richardson (1938) on the relation of *p*H to rate of penetration in fish; they suggest that ionised drugs have a low rate of diffusion across the intestinal membrane. Succinylsulphathiazole, which has been successfully used in the treatment of intestinal infection (Poth, Knotts, Lee and Inui, 1942; Poth and Knotts, 1942), is readily soluble in water but is not absorbed to any extent from the intestine. Presumably, as it is a fairly strong acid, it ionises, and so diffuses very slowly across the cell membrane.

If we seek relationships between structure and chemotherapeutic action in any blood infection, complications due to speed of absorption from the intestine must be controlled or eliminated. This may be achieved in two ways; firstly, by injection of the drug, or secondly, by administration of drug by mouth in such a way that a known and constant rate of absorption from the intestine is achieved. Administration of the drug in the food has been found to be the most convenient

method of control in routine testing in the mouse. This method, first used by Ehrlich and Hata (1911), has been particularly developed in the study of the sulphonamides by Bieter and by Marshall (Litchfield, White and Marshall, 1939; Bieter, Larson, Cranston and Levine, 1940).

When the disturbing effect of rate of absorption has been eliminated, relation of structure to distribution, metabolism and excretion has to be considered. Any change in chemical structure which is accompanied by a change in internal distribution will have fundamental bearing on overall chemotherapeutic activity and on toxicity; it will therefore affect the *chemotherapeutic index* of the drug $\left(\text{chemotherapeutic index} = \frac{\text{maximum tolerated dose}}{\text{minimum effective dose}}\right)$. The concentration of drug maintained in the blood and its toxicity for the host depend to a considerable extent upon its behaviour in the kidney, the main organ of excretion.

The functional unit of the kidney may be represented diagrammatically (Fig. 30) as consisting of a filter bed, the glomerulus, which is completely permeable to small molecules, and the tubules, which are capable of highly selective absorption and excretion. Formation of urine begins with separation, in the glomerular filter bed, of an ultrafiltrate which is identical in composition with the blood plasma, except for the absence of plasma proteins or other colloidal particles to which the filter bed is impermeable. Certain substances, for example glucose, which are invariably present in the glomerular filtrate, do not appear in the final urine. They must therefore be reabsorbed as the urine passes down the tubules. Other substances, such as phenol red, appear in the urine in higher concentration than can be accounted for by glomerular filtration (Marshall and Vickers, 1923) and are excreted by the cells of the tubular epithelium. Provided that a drug does not exist in the blood in a colloidal state or is not bound to blood proteins, change in chemical structure will not affect the rate of glomerular filtration, but may affect the rates of reabsorption or active excretion by the tubules. In order to study the relative contributions of these two processes,

it is necessary to have some method of measuring the rate of glomerular filtration and the amount of water reabsorbed from the glomerular filtrate by the tubules. The use of inulin, a physiologically-inert polysaccharide of molecular weight about 5000, as a guide to the rate of glomerular filtration and

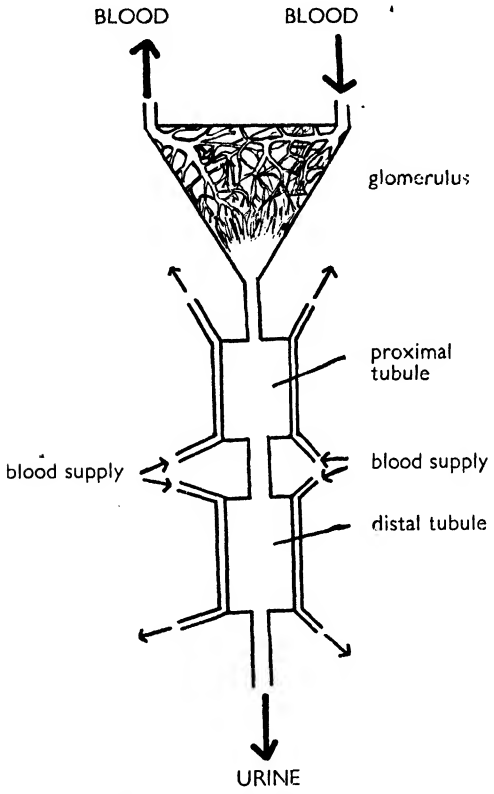


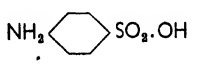
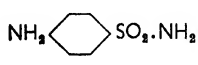
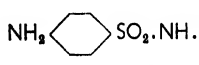
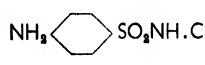
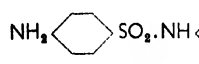
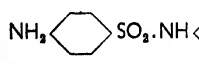
FIG. 30.—Diagrammatic representation of the kidney.

tubular reabsorption, has been developed by Shannon (1939), Smith (1943) and others. Inulin is completely filterable from the plasma by the glomerulus, but is sufficiently large to fail to pass back into the blood stream from the tubules or to be actively excreted by the tubules so that it is quantitatively excreted in the urine. Other diffusible substances present in the blood are filtered by the glomerulus at the same rate as inulin.

If they appear in the urine in the same ratio to inulin as they exist in the plasma, then they must, like inulin, pass through the tubules without reabsorption $\left(\frac{\text{drug}}{\text{inulin}} \text{ ratio} = \text{unity}\right)$. If the drug/inulin ratio is less than unity, the drug is being reabsorbed by the tubules from the glomerular filtrate. If the drug/inulin ratio is greater than unity then the drug

TABLE 25

Distribution of sulphonamides in the cat

Compound	Ratio: Concentration in tissue / Concentration in plasma/0·93			Volume of distribution, per cent. body weight	*p*Ka
	Muscle	C.S. Fluid	Brain		
	0·11	0·03	0·03	28·7	3·20
	1·07	0·68	0·73	98·2	10·43
	0·70	0·30	0·21	67·2	10·94
	0·16	0·03	0·04	29·9	3·52
	0·14	0·02	0·04	35·7	3·40
	1·23	0·07	0·15	165·0	7·85

From Fisher, Troast, Waterhouse and Shannon (1943).

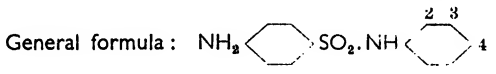
must be actively excreted by the tubules as well as filtered at the glomerulus.

Using this principle, Fisher, Troast, Waterhouse and Shannon (1943) made a most important study of the effect of change in structure among the sulphonamides on the rate of renal excretion. At the same time, they studied overall distribution, binding of drug by plasma protein, and its conjugation and metabolism within the animal. The results

of this study are so fundamental that they will be quoted at some length. Distribution studies were made on animals with the renal pedicles ligated so as to eliminate complications of excretion. A known amount of sulphonamide was injected intravenously, and blood samples were drawn and analysed after one or two hours and at the end of the experiment, at which time samples of cerebrospinal fluid, brain, lung, liver,

TABLE 26

Distribution of N^1-isocyclic derivatives of sulphanilamide in the cat

General formula: 

Substituent	Ratio: Concentration in tissue / Concentration in plasma/0·93			Volume of distribution, per cent. body weight	*p*Ka
	C.S. Fluid	Brain	Muscle		
None	0·18	1·06	0·80	125·0	9·60
4-NH_2	0·33	0·51	1·15	124·0	10·22
3-SO_2NH_2	0·14	0·19	0·94	146·0	8·23
4-SO_2NH_2	0·07	0·15	1·23	165·5	7·85
2-COOH	0·01	...	...	31·6	3·85
3-COOH	0·01	0·03	0·07	23·0	4·10
4-COOH	0·01	0·04	0·15	36·2	4·05
2-SO_2OH	0·01	0·06	0·10	23·9	3·40
3-SO_2OH	0·01	0·04	0·08	18·8	3·35
4-SO_2OH	0·02	0·04	0·14	35·7	3·40

From Fisher, Troast, Waterhouse and Shannon (1943).

pancreas, muscle and sciatic nerve were also taken. The extent to which each sulphonamide was bound to plasma protein was determined by dialysis of plasma samples using cellophane membranes. Tables 25 and 26 give a summary of observations on distribution in the cat.

It is at once obvious from the tables that there is a relationship between *p*Ka and distribution. The *p*Ka is the negative $\log_{10}$ of the acid dissociation constant and is numerically equal to the *p*H of a solution in which the drug

is 50 per cent. ionised. The relationship between *p*Ka, *p*H and percentage ionisation among acids is indicated by Fig. 31.

The distribution of sulphanilamide (*p*Ka = 10·43) is that of a substance capable of diffusing into all tissues and, in

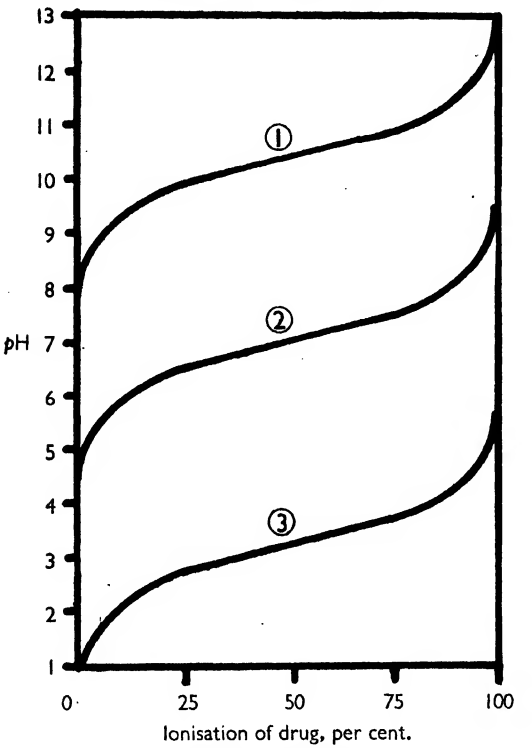


FIG. 31.—Relation between *p*K*a*, *p*H and percentage ionisation for acidic drugs.

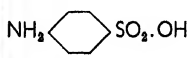
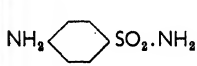
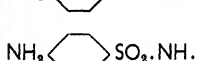
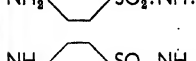
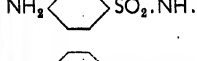
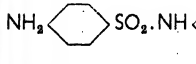
Curve (1) *p*Ka = 10·43 (sulphanilamide)
Curve (2) *p*Ka = 7·0
Curve (3) *p*Ka = 3·2 (sulphanilic acid)

addition, capable of being localised by some. This conclusion is reached because the volume of distribution of sulphanilamide is in excess of the water content of the body. Were sulphanilamide freely diffusible throughout the body and not localised in any tissue, its volume of distribution would be approximately 65 per cent. of the body weight (observed 98·2 per cent.) and

the tissue/plasma distribution ratios would be lower than those given in the table, *e.g* 0·75 for muscle. In the case of a drug confined to extracellular fluid, the volume of distribution should be about 25-30 per cent., and the tissue/plasma distribution in the range 0·0 to 0·2. The results quoted in Tables 25 and 26 indicate that the compounds with a high degree of dissociation (low *p*Ka) were largely confined to extracellular

TABLE 27

Excretion of sulphonamides in the dog

Compound	Per cent. filterable in Plasma	Drug/creatinine clearance ratio	Excretion ratio	*p*Ka
	98	0·89	0·91	3·20
	90	0·27	0·30	10·43
	89	0·63	0·71	10·94
	92	1·35	1·47	3·52
	34	2·23	6·29	3·40
	56	1·34	2·39	7·85

From Fisher, Troast, Waterhouse and Shannon (1943).

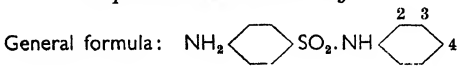
fluid and were almost incapable of passage across the blood-brain barrier; sulphonamides with *p*Ka range 7·85 to 10·22, were able to pass across cell membranes and were extensively localised within the cells. It is apparent, however, that structural factors as well as *p*Ka have an important effect, otherwise sulphanilamide and sulphanilylethanolamide would give almost identical figures. This is shown also by distribution studies made on *p*-aminobenzoic acid with *p*Ka 4·6. This acid, despite its low *p*Ka, passed readily across cell membranes and across the blood-brain barrier. This result must be related

to the fact that *p*-aminobenzoic acid plays some special part in the economy of the cell so that a special mechanism might be expected to exist for its active transport across cell membranes.

Tables 27 and 28 summarise observations made on the renal excretion of the same series of compounds in dogs. Shannon has used the drug/creatinine rather than the drug/inulin excretion ratio, but the significance is the same. The clearance ratio is the ratio drug/creatinine actually observed, but since

TABLE 28

Excretion of N^1-isocyclic derivatives of sulphanilamide in the dog

General formula: 

Substituent	Clearance ratio	Per cent. filterable in Plasma	Excretion ratio	*p*Ka
None	1·0	28	3·57	9·60
4-NH_2	0·36	65	0·55	10·22
2-$SO_2.NH_2$	0·08	35	0·22	?
3-$SO_2.NH_2$	0·73	38	1·92	8·32
4-$SO_2.NH_2$	1·34	56	2·39	7·85
2-COOH	0·77	12	6·42	3·85
3-COOH	1·84	15	12·26	4·10
4-COOH	1·67	34	4·91	4·05
2-SO_2OH	2·22	16	13·90	3·40
3-SO_2OH	2·30	24	9·58	3·35
4-SO_2OH	2·23	34	6·29	3·40

From Fisher, Troast, Waterhouse and Shannon (1943).

a certain proportion of the drug present in the plasma is in a non-diffusible form, this ratio has to be corrected for drug binding, and the excretion ratio is a true measure of functional processes going on in the kidney.

The data indicate that sulphanilic acid is filtered at the glomerulus and then passed down the tubules without much reabsorption by the tubules, whereas sulphanilamide is reabsorbed to a considerable extent. The three compounds, sulphanilylglycine, sulphanilylsulphanilic acid and sulphanilylsulphanilamide, are actively excreted by the tubules in addition

to being filtered at the glomerulus. The relation of behaviour to *p*Ka is now no longer obvious, since sulphanilic acid (*p*Ka 3·20) is excreted in quite a different way from sulphanilylsulphanilic acid (*p*Ka 3·40).

The difficulty of formulating a coherent theory to cover the relation between structure, *p*Ka and renal excretion is further exemplified by the results of Fisher *et al.* (1943) summarised in Table 28.

Höber (1940, 1945) has drawn attention to the importance of a hydrophilic-hydrophobic structure and lipoid solubility as factors in the behaviour of a drug in the kidney, but the exact relation of chemical structure, physiochemical characteristics and excretory mechanism is far from clear. On the question of distribution within the body the position is rather less obscure, as we have seen in the case of the sulphonamides.

Rate of excretion may be altered also by simultaneous administration of a second drug, for example the rate of excretion of penicillin may be considerably lowered by simultaneous administration of sodium - *p* - aminohippurate (Beyer, Peters, Woodward and Verwey, 1944). In the clinical application of penicillin, the maintenance of a suitably high blood concentration of penicillin has been a major problem. The realisation that the normal high rate of urinary excretion of penicillin might be lowered by simultaneous administration of another substance has led to the introduction of caronamide (4′-carboxyphenylmethanesulphonanilide). This drug, taken by mouth, blocks tubular excretion of penicillin so that the plasma concentration of penicillin resulting from a single injection is two to seven times normal (Strauss, Richburg, Saba and Alexander, 1947; Crosson, Boger, Shaw and Miller, 1947). Administration of acid or alkali so as to create an acid or an alkaline urine may also alter the rate of excretion (Andrews and Cornatzer, 1944; Beyer, Peters, Patch and Russo, 1944).

Effect of structure on the metabolic fate of drugs

The results quoted above make it clear that change in chemical structure within a series of related compounds may

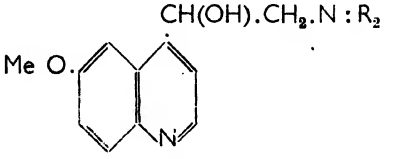
be accompanied by extensive alteration in physiological distribution, so that without any change of *in-vitro* bacteriostatic activity in a series of drugs, the chemotherapeutic index may vary within wide limits. In addition to the effect on distribution, alteration in structure may change the degree of conjugation or metabolism of drug in the animal.

In man, sulphonamides are acetylated to some extent in the N^4 position, and the N^4 acetylsulphonamides are inactive as bacteriostatic agents. The effect of change of structure upon degree of acetylation was examined by van Dyke, Tupikova, Chow and Walker (1945), but no correlation could be found between structure, *p*Ka, solubility, plasma binding and conjugation. Sulphonamides were not acetylated in the dog (Fisher *et al.*, 1943). Thus, owing to species difference in distribution or metabolism, results obtained with one animal may not be applicable to another or to man. Dobell and Laidlaw (1926) found that injection of emetine had no effect on intestinal infection with *Entamœaba histolytica* in the cat, but was curative in man. The quinine alkaloids are metabolised at different rates in the rabbit, chick and duck (Kelsey, Oldham and Geiling, 1945).

Knowledge of the metabolic fate of quinine has had useful application in guiding synthesis of compounds with greater chemotherapeutic activity. Quinine analogues such as 6-methoxyquinolylcarbinols of the general structure (I) were found to be effective in bird malaria (King and Work, 1940). When it was found that in animals quinine (II) was oxidised at the α-position of the quinoline nucleus to the corresponding hydroxy derivative (III), the possibility was suggested that antimalarial activity of (I) might be increased by prevention of oxidation through introduction of an α-substituent in the quinoline ring. A large series of such compounds have been synthesised and some were found to be much more active than quinine (Elderfield, 1946; Lutz *et al.*, 1946; Rapport, Senear, Mead and Koepfli, 1946).

The assumption was made in this work that increased antimalarial activity was a reflection of reduced metabolic destruction of the drug by host cells; this interpretation

might be worth extension. The close similarity between the oxidative enzymes of parasite and host indicates that a drug which is resistant to metabolic breakdown in the host may be particularly suitable as an enzyme inhibitor in the parasite, since it would also resist destruction within the parasite cell. The failure of many metabolite analogues as chemotherapeutic agents can probably be attributed to lack of adequate stability



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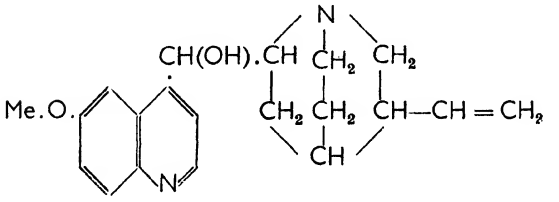

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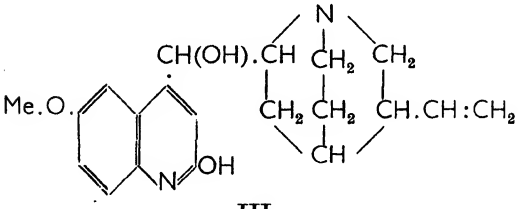


III

in the face of enzymic attack; knowledge of the metabolic fate of a drug in animals might suggest advantageous modifications.

The relation of structure to in-vitro *bactericidal activity: phenols and surface-active agents*

In the treatment of systemic infection, the blood concentration of drug required to achieve a 50 per cent. survival rate of infected animals is undoubtedly the most significant

measure of chemotherapeutic activity. However, the desire to probe as deeply as possible into the relationship of structure to bacteriostatic and bactericidal action necessitates strict limitation of variables. One group of variables can be eliminated by transfer of the parasite from its natural host to the culture medium and the test tube.

A general conclusion which can be drawn from all recent work on bacteriostatic and bactericidal action *in vitro* is that no substance which is active *in vivo* is inactive *in vitro*. To this must be added the qualification that a compound which is inactive *in vitro* may appear to be active *in vivo* because in the animal it is transformed into an active form. Such a change is exemplified in the pentavalent arsenicals, which are inactive *in vitro* but are transformed in the body to trivalent arsenicals. The latter show trypanocidal and bactericidal action both in the test tube and in the animal.

The converse of the above statement is certainly not true. Innumerable compounds are known which prevent bacterial growth in culture media, but which are useless in the body. This may result from two different causes. The more obvious is that the drug is highly toxic to the animal as well as to pathogen, so that no selective action is possible. The second cause arises from the fact that animal protein (tissue or blood) may interfere with the sterilising action of a drug, although in simple synthetic media the drug may be effectively bacteriostatic. The bacteriologist is therefore faced with the difficulty that the effect of change in structure as determined from *in-vitro* test may not be a guide to *in-vivo* activity, even when all complications arising from distribution and rate of excretion in the test animal have been eliminated.

One of the earliest methods developed for routine evaluation of germicides was that of Rideal and Walker (1903). In this method a drug is allowed to act on the organism in distilled water, and subcultures are made every two-and-a-half minutes up to fifteen minutes. The dilution of disinfectant which sterilises the suspension in a given time is divided by that dilution of phenol which sterilises the suspension in the same time. The figure so obtained is the *phenol coefficient* (for a

discussion on limitations of the coefficient, see Wilson and Miles, 1946; Reddish, 1937). By the use of this and similar methods, extensive studies have been made on the relation of structure to activity among the phenols (Harden and Reid, 1932; Richardson and Reid, 1940; Suter, 1941). In general, it may be said that the introduction into the phenol molecule of substituents such as alkyl, alkoxyl, alkylthiol and halogen increases the bactericidal activity as determined by the phenol coefficient method. The larger the alkyl or alkoxyl group, the greater is antiseptic action. These results suggested to some workers that the bactericidal action of phenols was

TABLE 29

Relation of phenol coefficient to surface activity in alkyl resorcinols

Substance	Phenol Coefficient (*E. typhosa*)	Surface Tension (1/10,000 dilution). (Dynes per cm.) (air/water)
Resorcinol . .	0·3	76
Propyl resorcinol .	5	73
Butyl resorcinol .	22	66
Amyl resorcinol .	33	60
Hexyl resorcinol .	50	54
Heptyl resorcinol .	30	43

Data from Frobisher (1927).

related to their surface activity, since, in any series of related phenols, the power of reducing surface tension increases with increase in the size of alkyl or alkoxyl group. Other workers considered that the action of phenols was related to oil/water partition coefficient (Frobisher, 1927; Richardson and Reid, 1940). The relation of maximum killing dilution to partition coefficient is shown in Fig. 32 for a series of $\alpha : \omega$-di-*p*-hydroxyphenylalkanes prepared by Richardson and Reid. The relation of phenol coefficient to surface tension lowering in a series of alkyl resorcinols examined by Frobisher is given in Table 29.

These results immediately throw us back to the theories of Overton and Meyer and pose the question of the nature of the

bacterial cell. The bacterial cell, like other plant cells, consists of a cell wall which is fairly rigid (Wamoscher, 1930) and which encloses the protoplasm. Studies of the effect of osmotic pressure indicate that the protoplasm is contained in a semi-permeable cell membrane (Lewis, 1941). When bacteria are placed in liquids of high osmotic pressure the protoplasm may shrink away from the cell wall; when placed in distilled water after growing in a highly osmotic medium, protoplasm

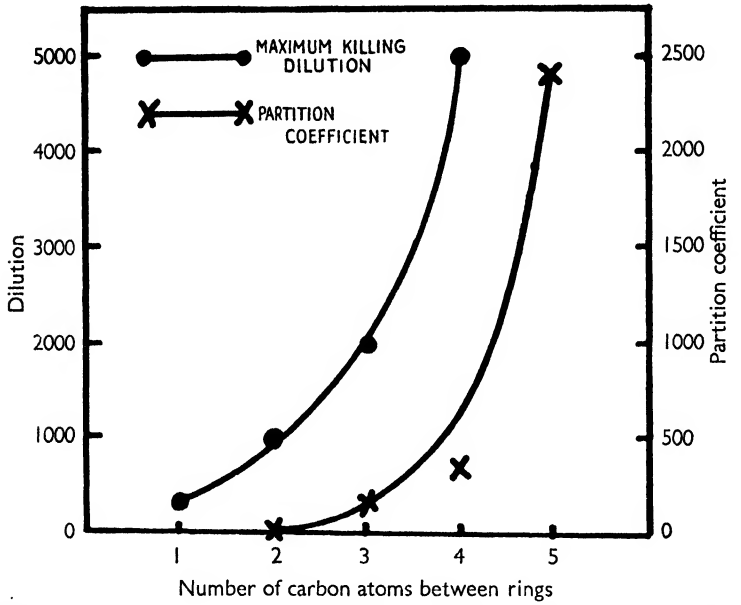


FIG. 32.—Relation of partition coefficient to maximum killing dilution among $\alpha : \omega$-di-*p*-hydroxyphonylalkanes. (Richardson and Reid, 1940.)

may be extruded at one end of the cell wall where it collects as a spherical mass (Dubos, 1945). Unfortunately scant quantitative data concerning the semi-permeability of the bacterial cell are available. Davson and Danielli (1943) suggest that the results of Collander (1937) on *Bacterium paracoli* (a late-lactose-fermenting strain of *E. coli*) indicate that its membrane is not an inert molecular sieve but is lipoid in nature.

There seems to be no adequate reason to assume that bacteria differ greatly from other cells in the nature of the

cell wall and plasma membrane; it is more probable that different species of bacteria differ widely among themselves, as do different other cells, and that all grades of permeability will be encountered; thus in the sulphur bacterium *Beggiatoa mirabilis* there is no evidence of any relation between permeability and partition coefficient (Table 30) and the cell wall behaves as a molecular sieve.

TABLE 30

Permeability of Beggiatoa mirabilis *to organic non-electrolytes*

Substance	Plasmolysis Threshold Concentration in mol/l.	Distribution Coefficient. Ether : Water
Urea	0·35	0·0005
Ethylene glycol	0·09	0·0068
Methyl urea	0·01	0·0012
Thiourea	0·075	0·0063
Glycerol	0·009	0·0011
Ethylurethane	0·015	0·6370
Lactamide	0·007	0·0018
Malonamide	0·007	0·0003
Dimethylurea	0·005	0·0116
Butyramide	0·0125	0·0580
Erythritol	0·001	<0·0001
Succinamide	0·0015	0·0002
Arabinose	0·0008	<0·0001
Diethylurea	0·003	<0·0185
Glucose	0·00055	<0·0001
Mannitol	0·00055	<0·0001
Sucrose	0·00020	<0·0001

Modified from Höber (1945). Data by Ruhland and Hoffmann (1925).

Analysis of red cell ghosts has indicated that the plasma membrane consists of lipoid and protein in the ratio 1 to 1·7. The lipoid fraction consisted chiefly of phospholipid and cholesterol (Parpart and Dziemian, 1940). The cytoplasmic membranes of *Bacillus megatherium* and *Bacillus cereus* have been examined in detail by Knaysi (1946), who concludes that in Gram-positive as well as in Gram-negative species the membrane is a combination of lipoid and protein. Indirect evidence for the complexity of cell membranes derives from the model experiments of Nirenstein (1920). By the use of a neutral oil containing added organic base and acid as a

membrane model, results were obtained on relative semi-permeability to dyes closely resembling those obtained on living cells. Neutral oil alone did not give comparable results. Osterhout (1940) also set up model membranes using a guaiacol mixture. Other workers have shown that phospholipids can form stable insoluble complexes with basic proteins (Schmitt and Palmer, 1940; Chargaff and Ziff, 1939). These results all suggest that the plasma membrane is likely to be a complex mosaic of fatty material and protein.

It would be possible to explain the increase in phenol coefficient within any one series of phenols as a reflection of the lipoid nature of the plasma membrane. This, however, does not help us to decide whether the increase in phenol coefficient, occurring as a series is ascended, is due to increase in the concentration of phenol reaching the cell enzymes, or whether the action is a detergent one on the plasma membrane. Considerable light has recently been thrown on this point by the fundamental observations of Hotchkiss (1944, 1946) and of Gale and Taylor (1946*a*, 1947*a*). The use of lysine decarboxylase to estimate manometrically the lysine content of washed cell suspensions of *Strep. fæcalis* showed Gale and Taylor that lysine could not be washed out of normal cells by saline or by distilled water. When cells were suspended in a solution of phenol of a concentration which would be expected to be bactericidal, lysine escaped rapidly from the cells; the same effect was found with cetyltrimethylammonium bromide, with Aerosol-AT or with tyrocidin. We are inclined to suggest, therefore, that the phenols and other surface-active bactericidal agents owe their *initial action* to their surface activity in virtue of which they plasmolyse the cell membrane. Such a mechanism would be consistent with the fact that bacterial cells may be rendered resistant to such drugs as sulphathiazole and penicillin, substances which we believe to act only by inhibiting cellular enzymic processes, but not to phenols, soaps, or detergents, substances which probably act initially by a non-specific physical disorganisation of the plasma membrane.

It might be expected at first sight that bactericidal power

should parallel surface activity, and that for each organism there would be a critical surface tension below which growth would become impossible; such is not the case. Frobisher (1926) found that *Staph. albus* was completely inhibited at a surface tension of 44 dynes per cm. produced by sodium glycocholate, but multiplied readily at a surface tension of 34 dynes/cm. produced by sodium oleate. Such a result is not unexpected. The belief that detergents act initially by disorganisation of the plasma membrane does not imply that any secondary form of interaction with cell proteins is excluded.

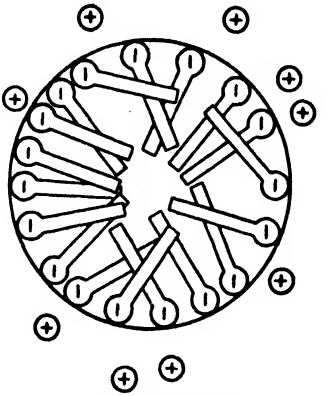


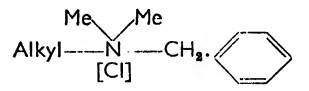
FIG. 33.—Detergent micelle of non-polar tails with polar heads. (Ralston, 1946).

In any one series of related detergents where the hydrophilic group remains unchanged and the hydrophobic group is increased in size, surface activity will increase with increase in molecular weight until a limiting size is reached. Beyond this limit, owing to the squeezing out effect exerted by water molecules in their endeavour to form hydrogen bonds with one another, and to the magnitude of the van der Waals forces between the hydrophobic chains, water solubility decreases rapidly and micelles are formed (see Fig. 33; Ralston, 1946). In such a series, complications due to change in the reaction between the polar group and cell proteins would be largely eliminated, and, up to the point of micelle formation, activity would be expected to increase with increasing chain length.

The effect of chain length on bactericidal effectiveness has been studied by Valko and DuBois (1945). In a series of aliphatic dimethyl-benzylammonium chlorides of the general structure shown, maximum potency was reached with the dodecyl or tetradecyl chain (Table 31).

TABLE 31

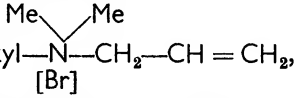
Effect of chain length on bactericidal action : cationic detergent

General Structure. 

Alkyl Substituent	Dilution killing in 10 minutes but not in 5 minutes	
	Staph. aureus	*Eberthella typhosa*
Octyl . . .	1 : 170	1 : 280
Decyl . . .	1 : 900	1 : 1000
Dodecyl . . .	1 : 20,000	1 : 16,000
Tetradecyl . .	1 : 21,000	1 : 17,000
Hexadecyl. . .	1 : 14,000	1 : 11,000
Octadecyl . . .	1 : 5,000	1 : 7,000
Octadeeenyl . .	1 : 29,000	1 : 10,000

From Valko and DuBois (1945).

In a homologous series of dimethyl-ethylammonium bromides the maximum potency was shifted towards compounds with a slightly longer alkyl chain, as shown in Table 32.

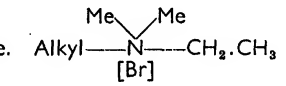
In the alkyl dimethyl-allylammonium bromides of the general structure  , maximum potency was reached with alkyl = C_{14} to C_{16}. In this case, however, the introduction of a double bond into the alkyl chain, which has so marked an effect in the two series tabulated above, had no effect on the minimal bactericidal concentration. It is obvious from these results that so long as the general molecular structure and the polar group are unchanged, additional CH_2 groups in the alkyl chain have a regular effect.

Bactericidal action, like surface activity, reaches a peak and then declines. These observations are consistent with the theory that initial action depends on surface activity. It does not seem possible at present to correlate the striking effect of such a small change as the introduction of a double bond with either surface activity or action on cellular proteins.

It should be remembered that while the results in any one series quoted above are consistent with the surface activity theory, they do not exclude other theories which may be able

TABLE 32

Effect of chain length on bactericidal action: cationic detergent

General Structure. 

Alkyl Substituent	Dilution killing in 10 minutes but not in 5 minutes	
	Staph. aureus	*Eberthella typhosa*
Dodecyl	1 : 7,700	1 : 5,800
Tetradecyl	1 : 8,000	1 : 12,000
Hexadecyl	1 : 20,000	1 : 12,000
Octadecyl	1 : 11,000	1 : 8,400
Octadecenyl	1 : 28,000	1 : 19,000

From Valko and DuBois (1945).

to account for the observed relationships in a consistent manner. When surface activity is present, and is sufficient to disorganise the plasma membrane and render it permeable to a detergent, the increase in activity with increasing numbers of CH_2 groups may be a reflection of increasing efficacy of the series as enzymic poisons or as protein denaturants. It is obvious then that even in a simplified system such as a bacterial suspension in the test tube, the variation in bactericidal action with structure within a single series of closely related compounds may be interpreted in a number of ways, all equally

consistent with known facts. What is more important is that all theories are open to confirmation or refutation by properly designed experiments where each variable is isolated and the effect of structural change upon it is examined. Thus there is need for a systematic study of the relation of change in structure *within a homologous series* of detergents to protein denaturation, to membrane permeability and to enzyme inhibition.

Alteration of the nature of the polar group has a much more profound effect on bactericidal activity than can be accounted for by change in surface tension. This may be related, firstly, to the complex nature of the plasma membrane, and secondly, to the nature of the interaction of detergent with cell proteins. Interaction of an ionisable detergent with plasma membrane may involve chemical reaction with some acidic or basic constituent of the membrane. The very complex nature of the interaction between surface films and detergents has been indicated by the work of Schulman and Rideal (Schulman and Rideal, 1937; Rideal, 1939; Rideal and Schulman, 1939). We are not in a position at present where we can find any close relationship between the nature of the polar group and the plasma membrane penetrant power of a detergent. Only one generalisation can be made, Gram-negative organisms are more resistant to the bactericidal effect of anionic detergents than are Gram-positive organisms, although both groups are susceptible to cationic detergents.

The relative insensitivity of Gram-negative organisms to anionic detergents is due, at least partly, to failure of these detergents to pass the plasma membrane. The enzymes of intact cells of *Escherichia coli* were shown to be relatively resistant to the inhibitory action of anionic surface-active agents, but enzyme preparations made by grinding the cells showed the same degree of sensitivity as did the enzymes in intact cells of a Gram-positive organism (Dyar and Ordal, 1946). Other cells may contain substances which neutralise the lytic effect of some surface-active agents (Ponder, 1946). Burdon (1944) and Dubos (1941) have suggested that

Gram-negative organisms have a different lipoid distribution from Gram-positive. Baker, Harrison and Miller (1941*b*) have shown that phospholipid may protect cells against detergents.

All these results taken together suggest that the ultimate cause of the bactericidal action of ionised detergents must be sought in some more specific mechanism than physical disorganisation of the plasma membrane by surface-active molecules. Valko and DuBois (1944) found that the " killing " action of surface-active cations could be prevented if, within a short time of the addition of cationic detergent, an equivalent of an anionic detergent was added to the cell suspension. Disorganisation of the plasma membrane has been shown by the work of Gale and Taylor (1946*a*, 1947*a*) to be an extremely rapid process. It seems reasonable to conclude that this disorganisation does not in itself constitute the lethal process, but enables the detergent to pass rapidly into the cell and combine with cell proteins. Provided that an anionic detergent is added soon enough, it can combine with the cationic detergent and so remove it from the cellular protein, thus the cell can be revived and temporary disorganisation of the plasma membrane does not do the cell any permanent injury. The results of Baker, Harrison and Miller (1941*a*) point in the same direction. They found that some detergents in suitable concentration stimulated cell respiration to a marked degree. Such stimulation probably reflects the increased permeability of the plasma membrane induced by a concentration of detergent which is non-toxic ; under such conditions, nutrient materials may be able to diffuse rapidly into the cell. Neutral detergents which cannot combine with carboxyl or amino groups of cellular proteins are particularly non-toxic and have been utilised by Dubos and Davis (1946) to speed up the rate of growth of *Mycobacterium tuberculosis*.

The nature of the initial combination of detergent and cell is also indicated by the effect of *p*H on the bactericidal power of detergents. Anionic detergents are more effective at low *p*H, cationic detergents at high *p*H (see Table 33). It has been suggested that the effect of change in *p*H is to change the

permeability of the plasma membrane, but in the case of the detergents, this seems unlikely, since the plasma membrane is probably rendered completely permeable by the detergent. Two other possibilities have to be considered, change in *p*H may affect the ease of denaturation of proteins by detergents, or it may affect the efficacy of the detergent ion as an enzyme inhibitor.

Proteins are ampholytes possessing potentially-ionisable acidic and basic groups. A detergent such as cetylpyridinium

TABLE 33

Effect of change in p*H on the bactericidal action of detergents*

Detergent	Concentration necessary to kill *Staph. aureus* in 5 mins.					
	*p*H 4	*p*H 5	*p*H 6	*p*H 7·2	*p*H 8·2	*p*H 9
Cetyl - dimethyl-benzylammonium chloride (cationic detergent)	$\frac{1}{100}$ does not kill in 15 mins.	$\frac{1}{100}$	$\frac{1}{300}$	$\frac{1}{800}$	$\frac{1}{5000}$	$\frac{1}{18000}$
	*p*H 4	*p*H 5	*p*H 6	*p*H 7		
Di-octyl sodium sulfosuccinate (anionic detergent)	$\frac{1}{3500}$	$\frac{1}{30000}$	$\frac{1}{40000}$	$\frac{1}{100}$ does not kill in 15 mins.	...	...

From Gershenfeld and Milanick (1941).

bromide, which in solution forms a pyridinium cation, would obviously tend to form a salt with an ionised protein carboxyl group; the anion of a detergent such as sodium lauryl sulphate would tend to form a salt with an ionised amino group. A neutral detergent, such as the ester of a polyhydric alcohol, would have no tendency to form a salt with either carboxyl or amino groups.

The effect of change in *p*H upon the tendency of anions or cations to form complexes with the carboxyl or amino groups of the bacterial cell was first fully discussed by Stearn and Stearn (1924, 1931). The equilibrium between cation and protein is represented in its simplest form in Fig. 34, where the cation

is designated as $\overset{+}{B}H$. The anionic form of the protein is represented as $P\overset{\ominus}{O}$, which with a hydrogen ion can form the undissociated acid $\overset{-}{P}O\overset{+}{H}$. The salt-like compound formed between protein and cation is designated as $\overset{-}{P}OB\overset{+}{H}$. This salt will have a tendency to dissociate in solution, and the concentrations of the various reactants will be influenced by change in *p*H as indicated in Fig. 34. The degree of salt formation and of ionisation will depend, at any one *p*H, on the dissociation constants of the reactants. Increase in hydrogen ion concentration (low *p*H) will favour formation of undissociated protein but will favour formation of dis-

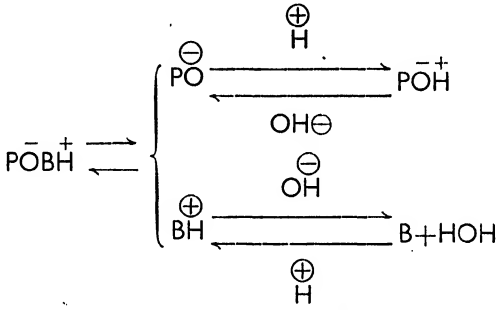


FIG. 34.—Equilibrium between protein and cationic detergent. Ionic charges ⊕, ⊖; formal charges +, −.

sociated base. Increase in hydroxyl ion concentration (high *p*H) will favour formation of dissociated protein but will depress ionisation of the base. The overall effect will depend upon the relative strengths of acid and base. Potential acidic groups of the protein molecule are not fully ionised at *p*H 7, but the cationic detergents are strong bases and fully ionised at this *p*H. Increase in *p*H will increase concentration of protein anion without affecting the concentration of cation, since the strongly-basic detergent will remain ionised over a fair range of *p*H. Because the concentration of anion is increased without a corresponding reduction in the concentration of cation, salt formation will be favoured, *i.e.* the bacterial cell will take up larger quantities of cationic detergent as the *p*H

is raised. This is reflected in the increasing bactericidal effectiveness of cationic detergents as *p*H is raised. Conversely, where the reaction involved is between the basic group of a protein and an anionic detergent, low *p*H will favour salt formation and therefore also bactericidal action (*cf.* Table 33).

Although the *p*H effect demonstrates that detergents combine as ions with oppositely-charged groups of the cell protein, it does not distinguish between generalised protein denaturation and enzyme inhibition as mechanisms of bactericidal action. Anson (1939) and Kuhn and Bielig (1940) have shown that detergents denature proteins, but the concentration of detergent required was very much higher than is required for bactericidal action. Few studies have been made on the effect of detergents in low concentrations on isolated enzymes; Marron and Moreland (1939) found that an anionic detergent had little effect on urease, while Kuhn and Bielig found that catalase was inactivated above *p*H 7 by cationic detergents. Baker, Harrison and Miller (1941*a*) found that bactericidal action paralleled inhibition of metabolism of intact cells with many detergents, but how far this inhibition was a cause and how far a result of some other process, it is impossible to say. Denaturation does not seem to be a specific enough effect to account for the great variation in activity with alteration of the ionisable " headgroup."

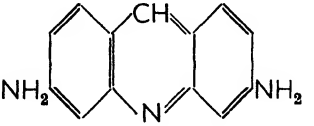
The fact that at neutral *p*H bacteria usually carry a negative charge (*i.e.* carboxyl groups predominate over amino groups) may explain in part the fact that cationic detergents are more effective at neutral *p*H than anionic detergents. The difference in susceptibility of Gram-negative and Gram-positive organisms to detergents cannot be explained entirely on the basis of difference in the numbers of free acidic and basic groups in the two types of organisms, since they also differ considerably in the nature of their lipoid constituents (Williams, Bloor and Sandholzer, 1939), and hence, probably, in the susceptibility of their plasma membranes to detergent action.

The phenols and detergents, which we have so far discussed as *in-vitro* antibacterial agents, have in common the feature

that they are very much less effective in the presence of body fluids than in simple culture media. This defect is associated with the fact that they react with all proteins. If *in-vitro* tests are made in the presence of blood or serum instead of in saline or simple synthetic media, phenols and detergents show little killing power. They are therefore largely restricted in use, and only applied externally on the animal body. A few phenols such as hexyl resorcinol are used in the treatment of intestinal helminthic infections ; here, no doubt, their successful use depends upon the fact that they are not absorbed too rapidly through the intestinal wall and so reach the worm in fairly high concentration. Phenols with a water solubility greater than 1 : 1000 are not suitable as anthelminthics (Jenkins and Hartung, 1943).

The relation of structure to activity among basic dyes

When the Stearns developed their theories regarding the effect of hydrogen ion concentration, they did so, not with respect to detergents, but to acidic and basic dyes. The bactericidal action of dyes was recognised by Ehrlich and by Stilling (see Chapter I), and their value in the treatment of local infections was utilised by Churchman (1912) and by Browning, Gulbransen and Thornton (1917). The acidic and basic dyes, unlike the phenols and detergents, can exert their antibacterial action in the presence of body fluids. The group of basic dyes which have been most fully examined from the point of view of relation of structure to activity are the acridines. Proflavine,



introduced by Browning, is as effective in serum as in simple media, but is less effective in blood.

Gale and Taylor (1946*a*, 1947*a*), when examining the effect of detergents upon the permeability of the bacterial cell, also examined gramicidin, sulphathiazole, acriflavine and penicillin,

all of which are effective bacteriostatic agents. None of these compounds altered cell permeability to amino acids. This fundamental observation at once distinguishes the detergents, which are bactericidal, from drugs which are, in suitable concentrations, bacteriostatic. Penicillin does, however, alter the function of the plasma membrane by inhibiting active transport of glutamic acid across the cell wall, but this is a specific inhibition of a metabolic process and in no way comparable with the non-specific action of phenols and detergents (Gale and Taylor, 1946*b* (for details see p. 272)).

A drug is generally regarded as bacteriostatic if, when it is washed away from the cell or neutralised in some other way, the cell is able to resume growth and multiplication. As shown by Valko and DuBois, the bacteriostatic action of a detergent may be reversed when an antagonist is added soon enough. A true bacteriostat, however, can be left in contact with cell suspensions at concentrations greatly in excess of the minimal bacteriostatic concentration without apparently causing any permanent injury. In this respect, the acridines appear to lie midway between antiseptics such as the phenols and bacteriostats such as penicillin.

The demonstration by Stearn and Stearn (1926, 1930) that acidic dyes are more effective at low *p*H and basic dyes at high *p*H indicated that dyes formed salts with the acidic or basic groups of proteins. Stearn and Stearn therefore suggested that among a series of related basic dyes, the dye which was the stronger base would be the more effective bactericidal drug. This suggestion has been proved to be substantially correct in many cases, particularly in the acridine series of drugs.

Albert, Rubbo, Goldacre, Davey and Stone (1945) synthesised the series of mono- and diaminoacridines set out in Table 34 and measured their bacteriostatic index, their effectiveness as surface tension depressants, their dissociation constants and their oil/water partition coefficients.

Gale and Taylor's observation that acriflavine does not disorganise the cellular permeability and Albert's demonstration that in a series of 5-alkylaminoacridines antibacterial activity

does not increase with increase in surface activity, indicate that the acridines, unlike detergents, do not act initially on the cell membrane. This belief is confirmed by the fact that while serum proteins almost completely inactivate the deter-

TABLE 34

Physical properties and bacteriostatic indices of acridines

Substance	Per cent. Ionised *p*H 7 to 7·2	Oil/Water partition coefficient	Surface tension depression (dynes/cm.) (air/water)	Molar Bacteriostatic Index* B.I.	*p*Ka
Acridine . . .	0·3	200	2	6	4·8
1-aminoacridine .	0·1	1200	7	4	4·2
2-aminoacridine .	88	5·2	0·2	21	8·2
3-aminoacridine .	3·0	90	1·5	8	5·8
4-aminoacridine .	7·4	55	0	9	6·2
5-aminoacridine .	100	1·2	0	25	9·9
5-methylaminoacridine	100	12	0	22	10·1
5-butylaminoacridine	100	16	1·5	14	9·8
5-cyclohexylaminoacridine	99	77	2·5	15	9·2
5-heptylaminoacridine	100	1000	18	15	9·7
5-dodecylaminoacridine	99	...	36	14	9·5
2 : 5-diaminoacridine	100	1	0	17	11·4
2 : 7-diaminoacridine	88	...	0	26	8·2
2 : 8-diaminoacridine	100	0·7	0	22	10
acriflavine . .	100	<0·2	0	22	12
1 : 9-diaminoacridine	0·1	...	...	0	4·3
2 : 9-diaminoacridine	44	...	...	9	7·2

From Albert *et al.* (1945).

* For a definition of the term " bacteriostatic index " (B.I.) the paper by Albert, Rubbo, Goldacre, Davey and Stone (1945) should be consulted. It will suffice here to say that a bacteriostatic index less than 10 indicates poor antibacterial action; a B.I. of 15, moderate antibacterial action, while a B.I. greater than 20 indicates good antibacterial properties, *e.g.* acriflavine has a B.I. on this scale of 22.

gents, they have little effect on acridine-induced bacteriostasis. It is evident also that there is little connection between oil/water distribution coefficient and activity, but that there is a relationship between *p*Ka and activity. Albert and co-workers have examined over 50 acridines with a *p*Ka of 7·8 or more.

All except three of these had a bacteriostatic index equal to, or greater than, 15, *i.e.* they were moderate to good bacteriostatic agents. Of 33 compounds with a *p*Ka below 7·8, not one had a B.I. greater than 15. This feature is particularly well exemplified by the group of diaminoacridines listed in

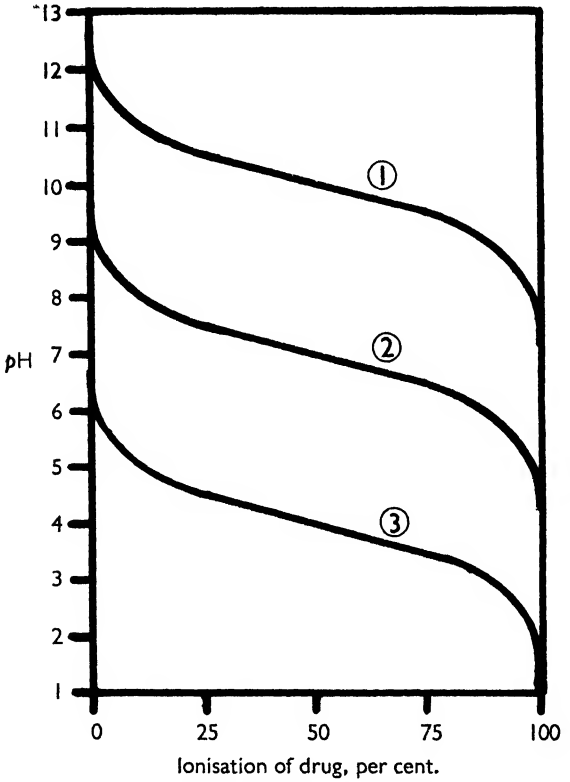


Fig. 35.—Relation between *p*Ka, *p*H and percentage ionisation for basic drugs.
Curve (1) *p*Ka = 10·0
Curve (2) *p*Ka = 7·0
Curve (3) *p*Ka = 4·0

Table 34. 2 : 7-Diamino and 2 : 8-diaminoacridine have *p*Ka 8·2 and 10·0 and bacteriostatic index of 26 and 22 respectively, whereas 2 : 9-diaminoacridine with a *p*Ka of 7·2 has a bacteriostatic index of only 9.

The relationship of *p*Ka to *p*H and percentage ionisation *for bases* is indicated in Fig. 35. This should be compared with

the corresponding figure (Fig. 31, p. 314) for the relation of *p*Ka, *p*H and ionisation among *acids*. It will be noted that the ionisation of a base with *p*Ka = 7 decreases as *p*H is raised, and also that at physiological *p*H an acid with a *p*Ka = 10 is unionised, whereas a base with *p*Ka = 10 is fully ionised.

Browning, Gulbransen and Kennaway (1919) showed that acridines were less effective in acidic than in alkaline media. This observation might be explicable either in terms of a permeability factor or in terms of competition between drug and hydrogen ion for anionic centres in the protoplasm. It has been frequently demonstrated (Osterhout, 1931 ; Eagle, 1945*a*) that many cells are less permeable to ions than to the related bases. As a medium is rendered acid, the percentage of basic drug in the unionised form decreases, and therefore the rate of penetration across the plasma membrane would be expected to decrease also. In the case of the acridines, however, this does not appear to be the most probable explanation, since, with the strongly-basic acridines, shift of *p*H from 7·5 to 5·7 causes a significant decrease in bacteriostatic index without change in ionisation as they are fully ionised. The effect of *p*H change cannot therefore be attributed to a change in the relative proportion of acridine ion to acridine base in the case of strongly-basic acridines. With weak acridine bases the same *p*H shift has no effect on bacteriostatic action, but degree of ionisation is greatly altered, from slight ionisation at *p*H 7·5 to almost complete ionisation at *p*H 5·7. This result suggests that the ratio of hydrogen ion to acridine ion is the controlling factor. Table 35 (from Albert *et al.*, 1945) gives the ratio of acridine cation to hydrogen ion at the minimal bacteriostatic concentration over the *p*H range 5·4 to 8·3.

Over this range of *p*H the concentration of acridine ion increases considerably more than one-hundredfold. At the same time the hydrogen ion concentration increases more than four-hundredfold, and the ionic ratio acridine$^{\oplus}$/H only changes threefold. In other words, an increase in hydrogen

ion concentration must be balanced by a corresponding increase in acridine ion concentration for bacteriostasis to occur. This is exactly what would be expected if acridine ions were acting as competitive inhibitors for a position on an enzyme surface normally occupied by hydrogen ions. On the basis of this theory, the other relationship observed by Albert and his colleagues also becomes understandable, that between *p*Ka or base strength and bacteriostatic activity. In a series of related acridines, as base strength increases, the percentage of drug present in the ionised form at physiological

TABLE 35

Relation of ionic ratio to bacteriostatic action of acridines

Substance	Ratio acridine ion/H ion at Minimal Bacteriostatic Concentration using *Escherichia coli*			
	*p*H 5·7	*p*H 6·8	*p*H 7·5	*p*H 8·3
5-aminoacridine *p*Ka 9·9	510	810	1000	1600
2 : 7-diaminoacridine *p*Ka 8·2	500	640	810	1300
	*p*H 5·4	*p*H 6·8	*p*H 7·5	*p*H 8·3
3-aminoacridine *p*Ka 5·8	190	285	641	...
3 : 7-diaminoacridine *p*Ka 6·2	210	580	590	...

From Albert, Rubbo, Goldacre, Davey and Stone (1945).

*p*H will also increase. When this reaches 100 per cent. and the base exists fully in the ionised form, any further increase in base strength will not be reflected in a corresponding increase in the ratio acridine ion/H ion at physiological *p*H, *i.e.* the maximum degree of bacteriostatic effectiveness will have been reached. Ionisation at physiological *p*H will be over 80 per cent. when the base has a *p*Ka higher than 8, and 100 per cent. when the base has a *p*Ka over 9. It will be found that in all the most effective acridines the *p*Ka is higher than 8.

Bacteriostatic index does not parallel exactly the *p*Ka (Table 34). This is to be expected, since the above theory

takes no account of the fact that, for any one structure, the critical ratio acridine ion/hydrogen ion will be a characteristic value dependent upon factors which cannot at present be assessed in a quantitative manner. Such factors include van der Waals' forces between the acridine ring system and the unionised neighbouring groups of the protein molecule,

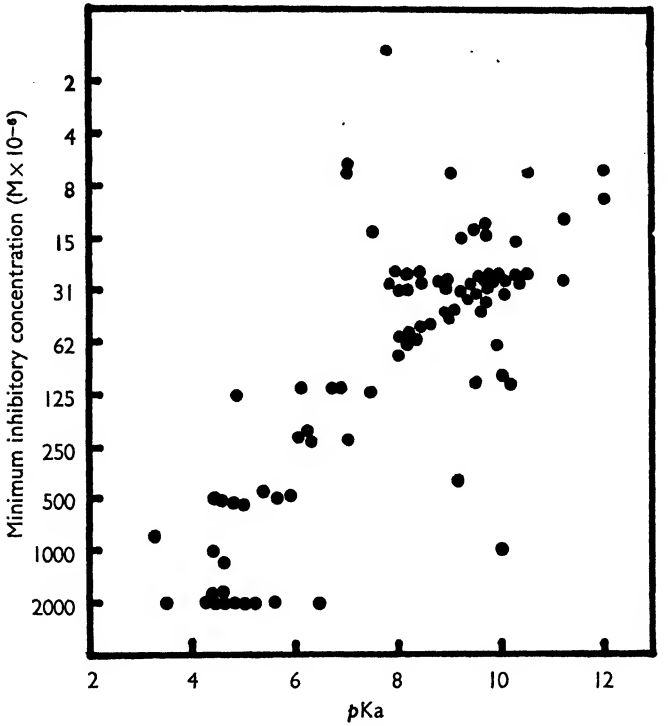


FIG. 36.—Relationship of antibacterial activity to base strength among acridines. Organism: *Strep. hæmolyticus*. (Albert, Rubbo, Goldacre, Davey and Stone, 1945).

or hydrogen bonding between secondary polar groups in the acridine ring and polar groups on the protein surface. Fig. 36 relates bacteriostatic action to *p*Ka for the whole series of acridines examined by Albert *et al.* (1945). There is considerable scatter, but if a best curve is drawn through the points and expanded to allow for experimental error, a considerable proportion of the points are found to lie on the area of curve.

Recent work, published since the construction of Fig. 36, reconciles apparent exceptions with the original hypothesis. 1-Hydroxyacridine, which possesses antibacterial activity greatly in excess of what would be predicted from its feeble ionisation, has been shown to owe its biological activity to an entirely different property, namely, its metal-chelating effect; this enables it to combine with important trace metals. Other apparent exceptions, the hydroxy-5-aminoacridines, exist to a large extent in the zwitterion form so that a high degree of ionisation does not result in a high concentration of the cationic form (Albert and Goldacre, 1948).

Relation between base strength and electronic configuration

Granted that there is a relation between base strength and bacteriostatic index, there remains the second question of the relation of base strength to molecular structure. A base in aqueous solution may be defined as a substance whose molecules are capable of forming covalent links with protons. The strength of a base will depend upon the equilibrium expressed in the equation:

$$B + H_2O \rightleftharpoons \overset{\oplus}{B}H + \overset{\ominus}{O}H$$

Any change in structure which will facilitate co-ordination of the proton will increase base strength. Co-ordination of the proton $\overset{\oplus}{H}$ will be decreased by decrease in electron density at the co-ordinating atom of the base. Therefore, introduction in a molecule of any substituent, which is electron-attractive in character, or which by some other means facilitates lowered electron density at the co-ordinating atom, will decrease base strength. Halogen is electron-attractive and will therefore tend to lower electron density if introduced as a substituent close to an amino group. In general, halogen-substituted bases are weaker than their parent molecules, as indicated in Fig. 37, where base strength is plotted against the number of chlorine atoms in halogen substituted anilines (Hall and Sprinkle, 1932). An effect on base strength of this type due to electron displacement by an electron-attractive group is known as an inductive

effect (Ingold, 1934). The influence of a substituent cannot always be expressed in terms of its inductive effect. Cyclohexylamine (*p*Ka 10·61) is a much stronger base than aniline (*p*Ka 4·6) and piperidine (*p*Ka 11·31), a much stronger base than pyridine (*p*Ka 5·21). In each of these cases, substitution of an aliphatic for an aromatic system apparently introduces

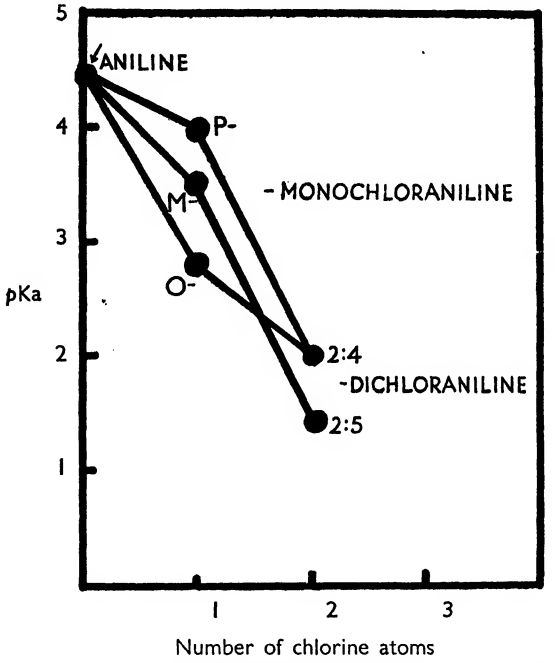


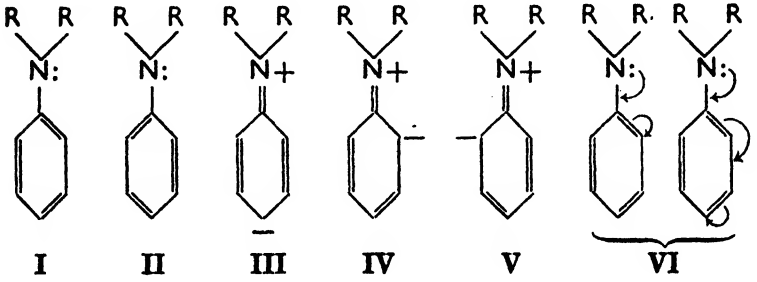
FIG. 37.—Inductive effect of chlorine in lowering base strength. (Hall and Sprinkle, 1932).

a factor other than the inductive effect; a factor which favours the left-hand side in the equilibrium

$$B + H_2O \longrightarrow \overset{\oplus}{B}H + \overset{\ominus}{O}H$$

The nature of this effect, the resonance or mesomeric effect, has been fully discussed by Pauling (1940) and by Branch and Calvin (1941), and a simple mechanical analogy has been introduced by Ingold (1938).

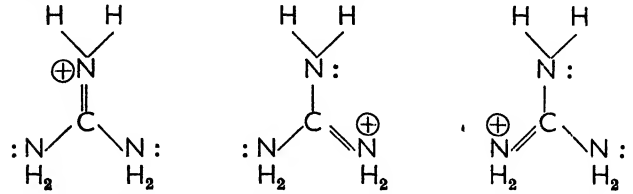
If resonance can occur in any molecular structure, that structure is more stable than any other closely-related structure in which resonance is either absent or less strong (Remick, 1943). In the case of an aromatic base of the aniline type, it is possible to represent the electronic structure by any one of a number of reasonable structures I to V as below :—



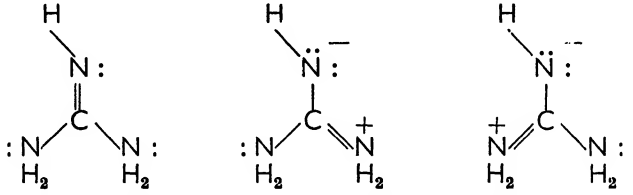
The quantum mechanical theory has demonstrated that it is impossible to define accurately the position of the electrons in a molecule at any instant of time, and that each of the above " unperturbed " structures represents only a theoretically-possible grouping of electrons. The normal state (which is the most stable) will not be represented by any one of these structures, but each one will contribute to the " ground state " in proportion to its relative probability. The anilinium ion, unlike aniline, does not possess on the nitrogen an unshared electron pair so that three quinonoid forms become impossible. The decreased base strength in aniline as compared to cyclohexylamine may then be postulated as due to the partial transfer of electrons in aniline from nitrogen to the conjugated ring system, as represented by the formulæ VI. In other words, it may be regarded as due to the fact that more extensive resonance is possible in aniline than in the anilinium ion and therefore the undissociated base form is more stable and probable than the ionised form of the molecule.

The existence of an opposite state to the above, where an ion is capable of freer resonance than the related base and is therefore the more stable form, might be inferred. Such a case

has been dealt with by Pauling (1938). The guanidinium ion can resonate between three equivalent structures :—

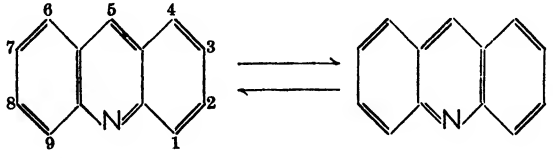


Resonance reaches a maximum when the contributing structures have nearly the same energy, thus in a case such as the above, where the three contributing forms are equivalent, the ion will be particularly stable. On the other hand, guanidine itself resonates between three structures which are not equivalent :—



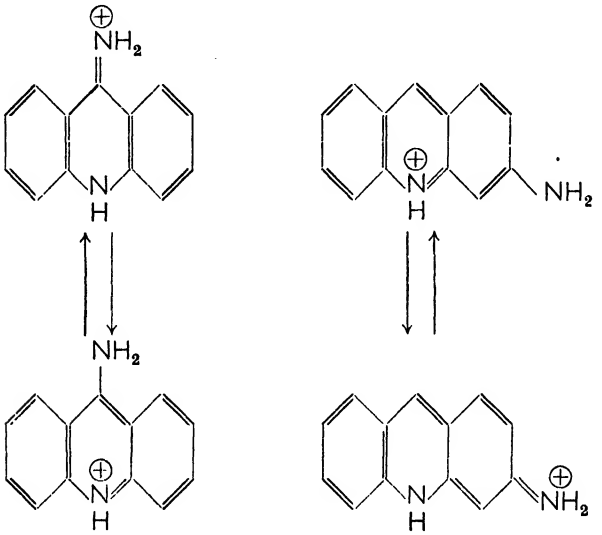
The ion will therefore be more stable than the base, and in solution guanidine will be a strong base, *i.e.* mainly in the ionic form.

The ideas which we have tried to express, and which are expanded at length in the textbooks referred to, have been utilised by Albert and Goldacre (1943) to trace a relationship between structure and base strength among the acridines. Acridine is a weak base (*p*Ka 4·8), comparable in strength to aniline and to pyridine, *i.e.* the base strength is reduced by resonance of the type indicated below :—



Introduction of an amino group in position 1-, 3- or 4- has little effect on ionisation, whereas 2-amino and 5-amino acridine are strongly ionised. Spectroscopic evidence indicates

that it is the ring nitrogen and not the amine substituent which ionises and is responsible for the increased base strength (Turnbull, 1945). The effect of the 2-NH_2 or 5-NH_2 must therefore be sought in terms of some effect upon the entire ring system. It appears to be explicable in terms of a heightened resonance effect in the ion to which the structures below would contribute.



The exaltation of basicity cannot be expected to be as great as in guanidine since the extra-ionic resonance is usually offset by the loss of nuclear resonance in one of the ionic structures.

Introduction of halogen in the acridine ring lowers base strength as indicated in Table 36. This too is explicable on the

TABLE 36

Inductive lowering of base strength by halogenation of acridines

5-aminoacridine	*p*Ka 9·9
5-amino-1-chloroacridine	*p*Ka 8·3
5-amino-2-chloroacridine	*p*Ka 9·0
5-amino-3-chloroacridine	*p*Ka 8·8
5-amino-4-chloroacridine	*p*Ka 8·4
2-aminoacridine	*p*Ka 8·2
2-amino-5-chloroacridine	*p*Ka 6·5

From Albert *et al.* (1945).

basis of the electronic concepts already set out. Chlorine, an electron-attractive element, will by its inductive effect, lower electron density on the ring nitrogen and so favour the left-hand side of the fundamental ionisation equation.

$$B + H_2O \rightleftharpoons \overset{\oplus}{B}H + \overset{\ominus}{O}H$$

Relation of structure to activity among sulphonamides

Several groups of workers at about the same time noted that there was a relationship between bacteriostatic activity and degree of ionisation among sulphonamides. In a discussion of the relationship of structure to activity it is convenient, therefore, to adopt the same approach as in the acridine series, firstly to consider the relation between *p*Ka and activity, and secondly to discuss the dependence of *p*Ka on electronic configuration.

The basic group in sulphonamides apparently plays a vital part in producing bacteriostasis, since any substituent on the primary amino group causes complete loss of activity. Variation in activity within a series of sulphonamides cannot, however, be attributed to change in the ionic strength of this group, since all active sulphonamides which are antagonised by P.A.B.A. have basic dissociation constants which are close to one another and to the value for the amino group in P.A.B.A., viz. $2{\cdot}5 \times 10^{-12}$. The majority of active sulphonamides also contain an acidic group with a measurable *p*Ka which may vary from *p*Ka 2 to *p*Ka 11. Fox and Rose (1942) noted that, with a variety of micro-organisms, sulphathiazole and sulphadiazine were about six hundred times more active than sulphanilamide, and that approximately six hundred times more P.A.B.A. was required to antagonise sulphathiazole and sulphadiazine than to antagonise sulphanilamide. The minimum bacteriostatic concentration of each drug was, however, antagonised by the same amount of P.A.B.A. This observation suggested that the active moiety was similar in each case, but that, as bacteriostatic power increased, a progressively larger proportion of each drug was in an active form. When the acid dissociation constants of the drugs were

measured and the percentage of each drug ionised at *p*H 7 was computed, the concentration of ionic form present in solution at the minimal effective concentration of each drug was found to be of the same order. The wide differences in the P.A.B.A./drug ratios contrasted sharply with the P.A.B.A./ ionised drug ratios which only varied over the range of 1/1·4 to 1/6·4 as indicated in Table 37. Fox and Rose suggested on the basis of these observations that the active forms of sulphonamides were the acid ionised forms, and to test this theory they measured the minimal bacteriostatic concentration

TABLE 37

Ionisation and effective concentration of sulphonamides

Drug	Minimum Effective Drug Concentration $M \times 10^{-6}$ (M.E.C.)	*p*Ka	Per cent. Ionised at *p*H 7·0	Concentration of Ionised Form at *p*H 7·0 $M \times 10^{-6}$	Minimum Amount P.A.B.A. required to antagonise M.E.C. $M \times 10^{-6}$	P.A.B.A. Drug Ratio	P.A.B.A. Ionised Drug Ratio
Sulphanilamide	2500	10·5	0·03	0·71	0·5	1 : 5000	1 : 1·4
Sulphapyridine	20	8·5	3·4	0·68	0·5	1 : 40	1 : 1·4
Sulphathiazole	4	6·8	61·6	2·46	0·5	1 : 8	1 : 4·9
Sulphadiazine	4	6·4	80·0	3·2	0·5	1 : 8	1 : 6·4

From Fox and Rose (1942).

of sulphanilamide at *p*H 6·8 and *p*H 7·8. Ionisation of sulphanilamide at *p*H 7·8 is increased tenfold over that at *p*H 6·8; in accordance with theory, it was found that the minimal bacteriostatic concentration at *p*H 7·8 was one-eighth of that at *p*H 6·8.

When other sulphonamides were examined from this point of view, strongly acidic sulphonamides were found to be less active than those of intermediate *p*Ka, and a plot of *p*Ka against *in vitro* bacteriostatic activity at physiological *p*H gave the parabolic curve indicated in Fig. 38 (Bell and Roblin, 1942). More recent work has tended to confirm this relationship (Walker, 1945; Cook, Heilbron, Reed and Strachan, 1945).

Cowles (1942) and Brueckner (1943) suggested that the parabolic form of the curve was related to the fact that the cell wall is impermeable to ions. If the ionic form is the active form, and the bacterial cell is impermeable to ionised sulphonamides, then maximum ionic concentration of any drug within the cell wall will be achieved when the *p*Ka of the drug is close to the *p*H of the medium used for test of

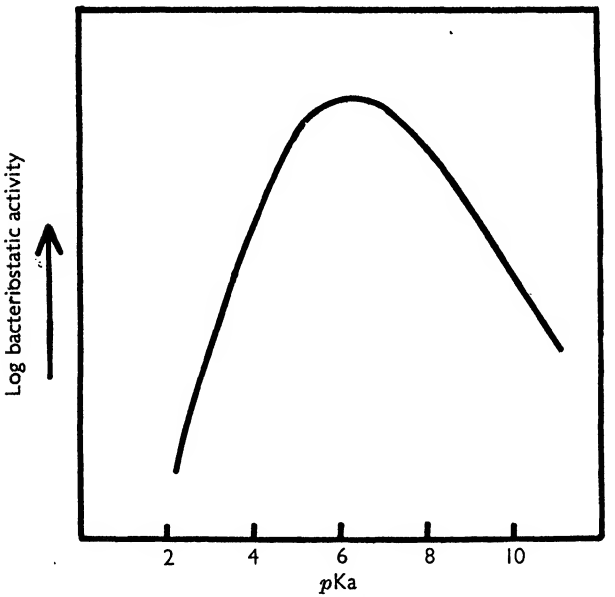


FIG. 38.—Relation of *in vitro* bacteriostatic activity to *p*Ka among sulphonamides. (Bell and Roblin, 1942).

bacteriostatic potency. Klotz (1944) has expressed the equilibria involved in sulphonamide action by a series of equations, assuming that the active form of the drug is the ionic form, and that drug action is brought about by combination of enzyme with drug. Equations set up on the basis of the law

[HD represents drug
P represents protein (enzyme)]

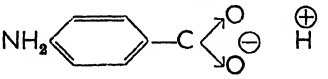
$$\overset{+}{H}+\overset{-}{D} \rightleftharpoons HD$$
$$\overset{+}{H}+P \rightleftharpoons \overset{+}{H}P$$
$$P+\overset{-}{OH} \rightleftharpoons P\overset{-}{OH}$$
$$P+D^{-} \rightleftharpoons PD^{-}$$

of mass action and the general concepts of ionisation, indicate that the maximum formation of drug-enzyme complex would occur when the *p*Ka approximates *p*H; also that a plot of *p*Ka against degree of formation of enzyme-inhibitor complex at any one *p*H would assume a parabolic form.

As outlined in Chapter V, the available evidence favours the view that sulphonamides act by displacement of P.A.B.A. from an active centre on an enzyme surface. Recent developments indicate that one of the reactions inhibited may be the synthesis of pteroylglutamic acid (folic acid). If this assumption is justified, the relationship between structure and activity among sulphonamides must be, firstly, a function of the concentration of sulphonamide at the site of action (a factor affected by cell permeability and by the extent of binding of sulphonamides by inactive proteins), and, secondly, a function of the relative dissociation constants of the enzyme-P.A.B.A. complex and the enzyme-sulphonamide complex.

At physiological *p*H the difference in activity between sulphanilamide and sulphathiazole cannot be attributed to difference in the amount of drug reaching the site of action; in both cases sufficient of the unionised drug would be present for equilibrium to be established. The ratio of unionised sulphanilamide to unionised sulphathiazole in equimolar solutions would be of the order of 2 : 1, whereas the ratio of their bacteriostatic activities is of the order of 1 : 300 to 1 : 600. The observed difference in activity must therefore be related to the relative dissociation constants of the sulphonamide-enzyme complexes.

P.A.B.A. is a fairly strong acid (*p*Ka 4·6) and is essentially completely ionised at physiological *p*H. The ionised molecule does not occur in the zwitterion form (Klotz and Gruen, 1945) and can therefore be represented as



A sulphonamide capable of ionisation at *p*H 7 may be expected to carry a similar charge in the ionic form and might be supposed to form a more stable complex with enzyme than the

corresponding molecule in the unionised form. The two competing processes, combination of enzyme with P.A.B.A. ion and with sulphonamide ion, may be represented diagrammatically as in Fig. 39. There is little justification beyond this formal structural resemblance of the ionised substrate and the ionised drug for the assumption that the ionised form is the only active form, or for the conclusion that combination of enzyme with unionised sulphonamide can be neglected. With sulphonamides which are only partially ionised at physiological *p*H, it is probably more correct to regard the ionised and unionised drug as both capable of forming inactive drug-enzyme complex. Diaminodiphenylsulphone, which cannot

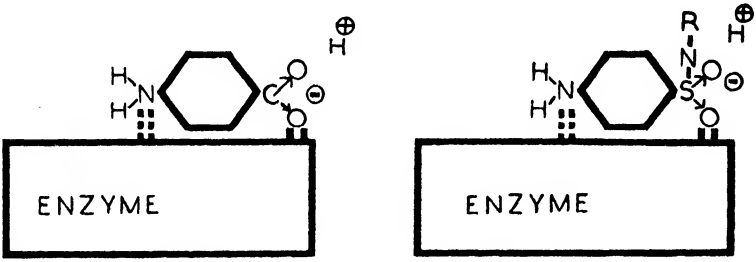


FIG. 39.—Diagrammatic representation of competition between a sulphonamide and P.A.B.A. at an enzyme surface.

undergo ionisation, is subject to the same type of antagonism by P.A.B.A. as the ionisable sulphonamides ; it is considerably more bacteriostatic than sulphanilamide, being nearly as active as the highly ionised heterocyclic sulphonamides.

Relation between acid strength and electronic configuration

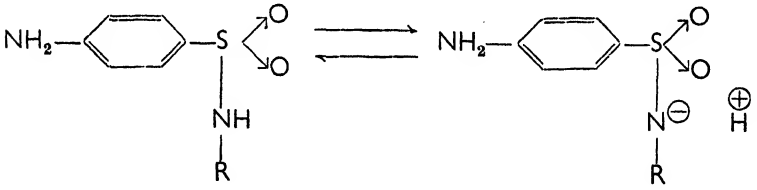
As indicated already, a plot of acid strength of sulphonamides against bacteriostatic index gives a parabolic curve. Maximum bacteriostatic activity occurs among those drugs where *p*Ka is close to the *p*H of the medium used for test. Such a relationship implies that if the *p*Ka of a sulphonamide can be predicted, then its *in-vitro* bacteriostatic index could also be predicted with a fair degree of certainty.

An acid in aqueous solution may be defined as a substance capable of dissociation according to the equation

$$DH + H_2O \rightleftharpoons D^{\ominus} + H.H_2O^{\oplus}$$

The degree of dissociation will depend on the attraction between $D^{\ominus}$ and $H^{\oplus}$, and this in turn will depend upon the electron density in the molecule at the point of attachment of the proton. Any decrease in electron density at this point will favour dissociation and so increase acid strength.

The only variable group in those sulphonamides which are antagonised by P.A.B.A. is the group R in the formula below.

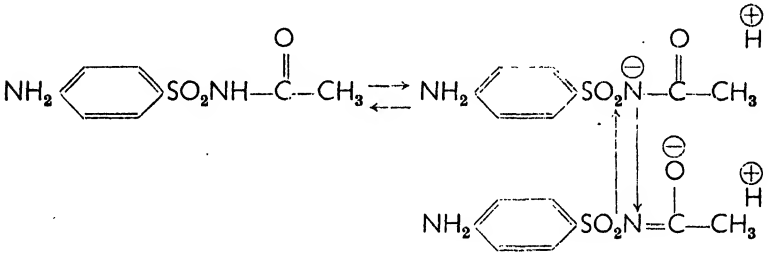


Alteration in R may alter acid strength by influencing the electron density at the amide nitrogen, the point of attachment of the proton. An electron-attractive substituent R will decrease electron density and increase acid strength, an electron-repulsive R group will have the opposite effect.

In addition to the inductive effect of the group R, the acid strength may be influenced by the possibility of resonance. If the ionic form of a sulphonamide is capable of resonance which is not possible in the same molecule in the unionised form, then the ionic form will be more stable than the corresponding unionised molecule. The drug will then be a stronger acid than might be expected from the inductive effect of the substituent R.

When these considerations are borne in mind, it is possible to predict the probable effect of any substituent R on the acid strength of a sulphonamide of the type $NH_2.Ph.SO_2.NH.R$, and hence to relate bacteriostatic activity to electronic configuration. Sulphanilamide (R = H) has a *p*Ka 10·4;

introduction of an alkyl group (slightly electron repulsive) results in a weaker acid, thus methylsulphanilamide ($R=CH_3$) has a *p*Ka 10·77. Aromatic rings are slightly electron attractive, thus phenyl sulphanilamide ($R = C_6H_5$) has a *p*Ka 9·6. Acyl groups are strongly electron attractive and acetylsulphanilamide ($R = COCH_3$) has a *p*Ka 5·4. In this case, ionisation is probably favoured by the possibility of resonance of the ionic form as well as by the electron-attractive character of the substituent group (Bell and Roblin, 1942; Walker, 1945).



Although it is thus possible to relate electronic configuration to *p*Ka, and *p*Ka to activity among ionisable sulphonamides of the general type $NH_2.Ph.SO_2.NH.R$, it is not possible to do this for other related drugs which are antagonised by P.A.B.A. Diaminodiphenylsulphone has already been mentioned as a notable exception; ketones such as *p* : *p*-diaminobenzophenone are also bacteriostatic and antagonised by P.A.B.A. It would seem that the active centre of the enzyme involved is designed to accommodate any molecule in which an aromatic amino group is separated from an electron-dative atom [*e.g.* $R{\rightarrow}\ddot{O}:$] by a suitable system of conjugated double bonds. Any substance of this type which can form an enzyme-drug complex with a low degree of dissociation is potentially an effective bacteriostatic agent.

Another interesting example of a relation between enzyme-inhibitor dissociation constant and electronic configuration of inhibitor is suggested by the work of Fildes and Rydon (1947) on methyltryptophans as inhibitors of tryptophan synthesis in *Eberthella typhosa*.

Relation of structure to activity among pantothenic acid analogues

Among sulphonamides a convenient simplification is introduced by the fact that variation in structure affects bacteriostatic action, *in vitro*, towards most micro-organisms in the same manner. Any attempt to relate structure to activity among pantothenic acid analogues meets with the serious difficulty that, although the majority are antagonised by pantothenic acid and therefore presumably act by the same mechanism, the relative activities of a series of compounds changes according to the organism used for *in vitro* test (Snell, 1946). This difficulty may be related to differences in the ability of different micro-organisms to split the amide linkage, or may be related to variation in the selective permeability of differing cell membranes. Even among sulphonamides, selective permeability effects can be utilised to explain the observed relationship between *p*Ka and activity as was done by Cowles (1942). Thus, while study of the relation of structure to activity in intact micro-organisms may throw out suggestive hints regarding the effect of the cell membrane, or may indicate a relationship between electronic configuration and stability of drug-enzyme complex, a full analysis of the relationship would necessitate isolation in a pure state of the enzyme systems involved, and a study of the kinetic relationships between the competing processes.

Relation of structure to inhibition of isolated enzymes

No chemotherapeutic drug can yet be assigned a specific role as inhibitor of one particular enzyme system, but study of the relation of structure to activity in isolated enzyme systems may indicate whether deductions made from experiments on that infinitely more complex system, the bacterial cell, are justified.

Few extensive investigations of the effect of a large series of related compounds on enzymes have been made, and of these even fewer are germane to the present discussion. Quastel examined a large series of acidic and basic dyes as inhibitors of urease and fumarase. Fumarase was readily inhibited by acidic dyes and somewhat less readily by basic

dyes; in contrast, acidic dyes were completely inert towards urease, but basic dyes were effective inhibitors. In both cases the appropriate substrate protected the enzyme to some extent against inhibitory dyes.

Comparison of dye inhibition of fumarase with dye inhibition of intact cells of *Escherichia coli* suspended in fumarate was particularly instructive. As shown in Table 38, a cell-free fumarase preparation extracted from *Escherichia coli* was inhibited by basic dye to the same extent as was the enzymic activity of intact cells, but an acidic dye (congo red) was a much less effective inhibitor of intact cells than of the isolated enzyme.

TABLE 38

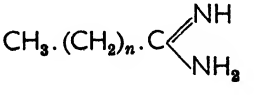
Inhibition by dyestuffs of fumarase extracted from E. coli *compared with inhibition of fumarase activity of intact cells* (E. coli)

Dyestuff $\left(\frac{1}{5000}\right)$	Percentage Inhibition	
	Cell Extract	Intact Cell
Congo red . . .	97	59
Methyl violet . .	82	88

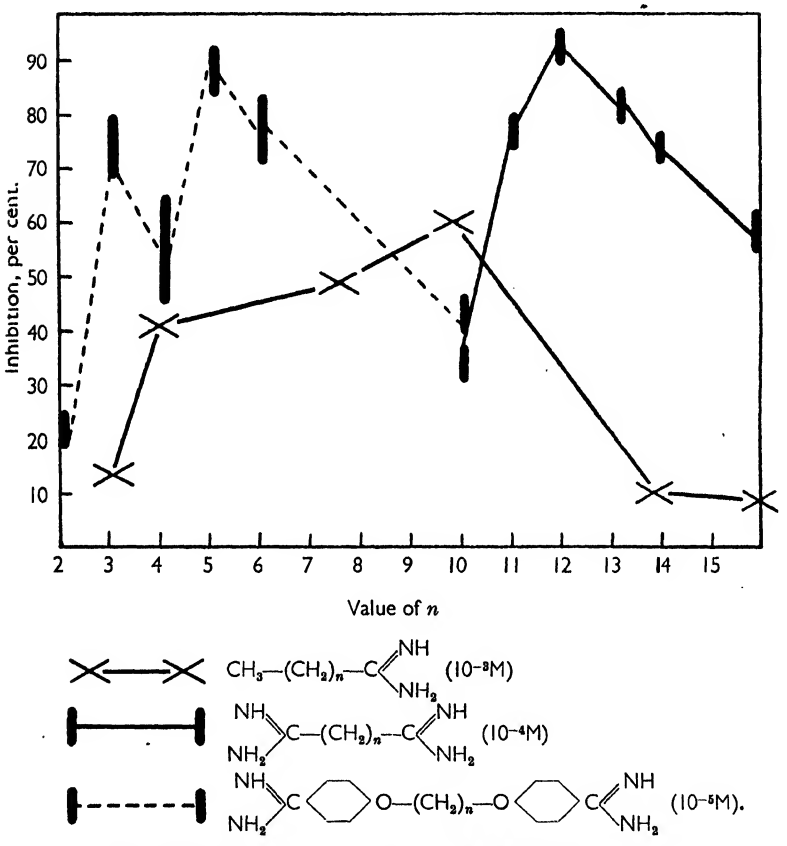
Data from Quastel (1931).

The conclusion derived from these experiments was that acidic dyes do not readily penetrate the cell wall. This conclusion was amply supported by examination of a series of 29 dyes as bactericidal agents. Basic dyes were toxic to *E. coli* and inhibited dehydrogenase activity of intact cells; acidic dyes were non-toxic and had also little effect as inhibitors of cellular dehydrogenase activity (Quastel and Wheatley, 1931; Quastel, 1931; Quastel, 1932).

Blaschko and Duthie (1945 *a* and *b*) examined a large series of amidines as inhibitors of amine oxidase. Monoamidines of the general type



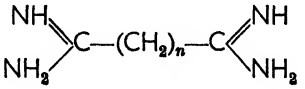
where n was 3, 4, 6, 8, 10, 14 and 16, were relatively poor inhibitors compared with some amidines, but the percentage inhibition increased regularly with increase in length of the



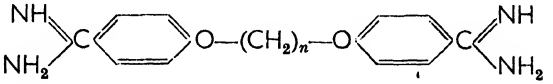
The vertical spread of individual points represents variation in successive experiments.

FIG. 40.—Inhibition of amine-oxidase by amidines. (Blaschko and Duthie, 1945).

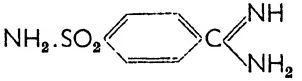
carbon chain up to $n = 10$ and then fell as indicated in Fig. 40. With alkyl diamidines of the general type



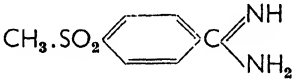
a greater inhibitory effect was observed; inhibition reached a maximum at $n = 12$ and then fell away as before. Aromatic amidines of the general type



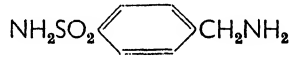
were particularly powerful inhibitors and showed a similar peak of activity, in this case at $n = 5$. Similar inhibition experiments with diguanidines and di-isothioureas showed that, with these compounds also, inhibition increased to a peak as the molecular weight increased, and then declined. Two closely related amidines,



and



differed considerably in their action. The first at a concentration of 10^{-3}M produced 57 per cent. inhibition, the second had no inhibitory action. The corresponding amines



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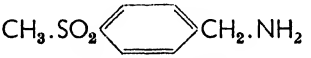


were readily oxidised by amine oxidase.

Among the alkyldiamidines, increased formation of enzyme-inhibitor complex with increasing chain length up to a maximum at $n = 12$ (Fig. 40), is paralleled by increased affinity of substrate for enzyme among the related alkyldiamines (Blaschko and Duthie, 1945*b*). The compounds tested were of the general structure

$$NH_2.CH_2(CH_2)_n.CH_2.NH_2,$$

where n was 4, 6, 12, 14 or 16. The amines with $n = 4$ or 6 had no affinity for enzyme; the amines of higher molecular

General formula. Ar—CHOH—N(R)(R).

Phenanthrene = Ar

Tetrahydrophenanthrene = Ar

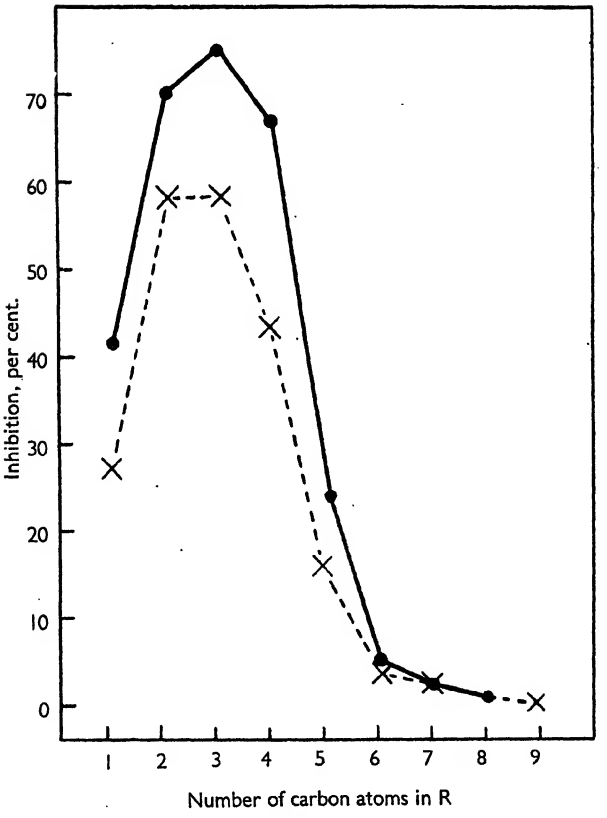
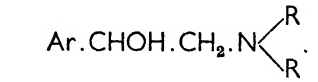


FIG. 41.—Inhibition of human plasma cholinesterase by phenanthrene-9-aminoalcohols and by tetrahydrophenanthrene-9-aminoalcohols. Concentration 5×10^{-7}M. (Wright, 1946).

weight had affinity for enzyme and were oxidised. Both rate of oxidation and affinity were maximal with $n = 12$, in other words, both natural substrate and inhibitory substrate analogue " fitted " the enzyme most effectively at the same chain length ($n = 12$).

Several homologous series of aromatic amino alcohols of the general type



have been investigated by Wright (1946) as inhibitors of red cell cholinesterase and plasma cholinesterase. Although the two enzymes were tested on the same substrate they showed considerable differences in response to inhibitors. Among a series of phenanthrene-9-aminoalcohols and tetrahydrophenanthrene-9-aminoalcohols, progressive increase in size of the dialkylamino group resulted in increased inhibition of plasma cholinesterase from dimethylamino to dipropylamino followed by rapid fall in activity (see Fig. 41).

Red blood cell cholinesterase was less easily inhibited, so that the same series could not be tested above diamylamino owing to solubility limitation. Up to this point activity increased steadily with increase in molecular weight (see Fig. 42). Reduction of the alcoholic hydroxyl group to a methylene group, oxidation to a keto group, or replacement by chlorine all decreased inhibitory action, but separation of the hydroxyl group from the aromatic system by a methylene group as in

enhanced inhibitory activity.

Progressive change in inhibitory activity with increase in chain length has been demonstrated also for alkylated succinic acids acting on succinic dehydrogenase, and for amino acids as inhibitors of arginase (Franke and Siewerdt, 1944; Hunter and Downs, 1945). For full analysis of the relation of structure to inhibitory action it is necessary, as shown by Hunter, to

distinguish between competitive and non-competitive inhibition, since one compound may act in both ways and change of structure may alter one type of inhibition in a different way from the other. The dangers inherent in the use of crude enzyme preparations have also been emphasised by these authors.

The problem of variation of rate of enzyme catalysis with change in substrate structure is even more complex, but as

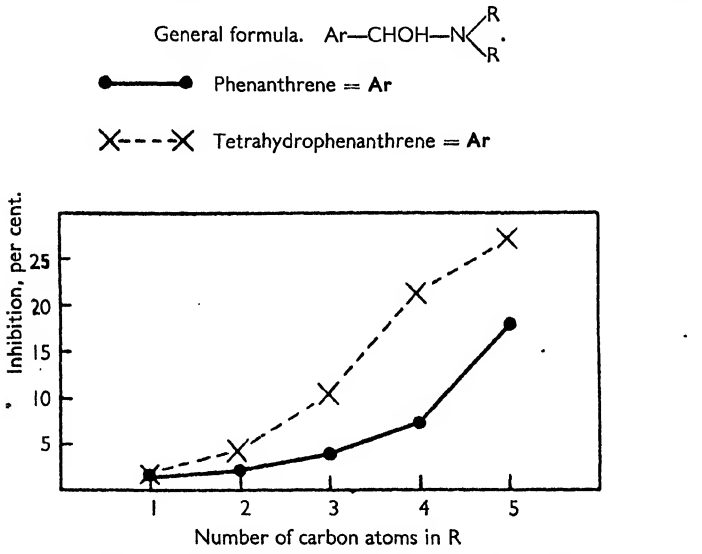


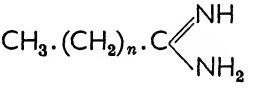
FIG. 42.—Inhibition of red cell cholinesterase by phenanthrene-9-amino-alcohols and by tetrahydrophenanthrene-9-aminoalcohols. (Wright, 1946).

shown by Blaschko and Duthie (1945 *a* and *b*) it has much in common with the simpler problem of relation of structure to inhibitory action. The controlling factor, so long as the electronic nature of the group undergoing chemical change is unaltered, is probably the dissociation constant K*s* of enzyme-substrate complex. In a series of alkyl-β-glucosides ease of hydrolysis by β-glucosidase was found to parallel K*s* (Pigman, 1944).

These results indicate that formation of enzyme-inhibitor complex is a highly specific process which can account for

many of the observed variations in bacteriostatic action. The increasing stability of enzyme-inhibitor complex as molecular weight is increased to an optimum is particularly noteworthy. There has been a tendency to ascribe change in activity in a homologous series to change in distribution, rather than to change in intrinsic action (Barger, 1930). These investigations show conclusively that the parabolic form of the molecular weight/activity curve in pharmacology may be more closely related to change in intrinsic activity at an enzyme centre than hitherto realised. The importance of molecular weight as one factor in overall chemotherapeutic activity has been emphasised (Work, 1940). How much of this importance can we attribute to selective distribution and how much to increased association of inhibitor with enzyme?

In Blaschko's inhibition experiments, the observed rise and fall in activity within a homologous series seems to involve two factors. Among monoamidines of the general type



increase in the value of n would result in increased van der Waals' forces holding together enzyme and poison. This increase is effective up to $n = 10$; somewhere between $n = 10$ and $n = 14$ there occurs a sudden fall in activity which can be compared to the sudden fall in bactericidal activity within a homologous series of detergents. At some critical chain length, the combination of mutual attraction between long chains and the forcing-out effect exerted by hydrogen bonding between water molecules causes micelle formation to take place (*cf.* p. 325). The peak of activity which occurs among the aliphatic diamidines at $n = 12$ cannot be explained on the same basis, since micelle formation would not be expected to occur until n was at least twice as large as in the monoamidines. The explanation must reside rather in an optimum degree of fit at a certain chain length. Blaschko's observation that, among aliphatic diamines, maximum rate of oxidation by amine oxidase occurs at $n = 12$ bears out this

view. No doubt increased association of enzyme and inhibitor over the range $n = 10$ to $n = 12$ is reinforced by increase in the van der Waals' force, but beyond $n = 12$ further increase in non-specific attraction, due to increased molecular weight, is insufficient to overcome the more dominant factor, degree of fit.

We have already remarked that the bacterium in the test-tube is a simpler system than the intact infected animal. It would be encouraging, therefore, if the intellectually satisfying results reported by Blaschko could be linked with *in-vitro* antibacterial activity. An attempt to work backwards in this way is a salutary reminder that we have deliberately simplified our system to the utmost. Fuller (1942) has estimated the *in-vitro* activity of the same amidines against bacteria. He found that serum caused an increase in the activity of shorter-chain drugs and a decrease in that of longer-chain drugs (protein binding ?). Monosubstituted amidines were more strongly inhibited by serum than diamidines. The diamidines had not reached their maximum activity against Gram-positive organisms by $n = 18$, but reached their maximum activity against Gram-negative organisms at $n = 13$.

Conclusion

Although it may be unprofitable to attempt to relate structure to overall chemotherapeutic activity, an analysis of the factors involved and of the effect of change in structure on each of these factors does provide useful evidence concerning the mode of action of drugs. A suggestive relationship between structure and activity may indicate a profitable line of investigation ; thus, knowledge of the relation of bactericidal action to surface activity among phenols and detergents suggested a more direct experimental attack. Direct investigation demonstrated that these substances damaged the semipermeable plasma membrane. The very striking difference in response of Gram-positive and Gram-negative organisms to bactericidal and bacteriostatic agents suggested that in Gram-positive organisms the protein moiety of the lipoprotein plasma membrane might have fundamental metabolic

functions. Direct investigation showed that Gram-positive organisms possessed a special transport mechanism for conveying amino acids into the cell.

When a drug is found to inhibit a particular enzyme, an examination of the relationship between structure and inhibitory action on the isolated enzyme may indicate whether it is reasonable to suggest that the drug owes its *in vivo* activity to inhibition of the same enzyme. For example, Mann and Keilin (1940) showed that sulphanilamide was an extremely effective inhibitor of carbonic anhydrase, but that nevertheless the antibacterial action of sulphonamides could not be attributed to this inhibition, since N-alkyl sulphonamides, which were effective inhibitors of carbonic anhydrase, were ineffective against bacteria.

EPILOGUE—CHEMOTHERAPY AS A SCIENCE

Man is, by nature, speculative, he is forever asking, how ? why ? First he collects a few facts, then he knocks together a scaffold on which to hang these facts and calls it a *Theory*. Periodically, as new facts are collected, additions and alterations are made to the scaffold of theory. At first it is no more than a mental convenience, a small concession to those troublesome genii, How and Why ; an aid to an imperfect memory and of little help in the design of new experiments or in the search for new facts. These crop up in spite of, not because of, the theoretical framework. As the body of fact expands, the scaffold of theory assumes a more solid form and a more definite shape. Eventually theory begins to outrun fact, and begins to guide the whole trend of research ; it is no longer a mental convenience but an essential part of an organised body of knowledge which has become a science.

In chemotherapy the transition state has been reached ; theory has overtaken fact and has begun to shape the future. In the process, it has inevitably drawn upon the more mature sciences, physical chemistry, organic chemistry and biochemistry, and in its turn it now has something to offer to

those sciences and to pharmacology. Metabolite analogues are not merely potential bacteriostatic agents; they are tools for the further unravelling of the steps of intermediary metabolism. Drug resistance is not only a headache for the clinician; it is a tool for the study of acquired character, inheritance, differentiation, and the relation of gene to environment.

* * * * * *

"*To invent without scruple a new principle to every new phenomenon, instead of adapting it to the old; to overload our hypothesis with a variety of this kind; are certain proofs, that none of these principles is the just one, and that we only desire, by a number of falsehoods, to cover our ignorance of the truth.*"

—David Hume, *A Treatise of Human Nature*, Book II, Pt. 1, Sect. III.

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