ANNUAL REVIEW OF MICROBIOLOGY

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CHARLES E. CLIFTON, *Editor*
Stanford University

SIDNEY RAFFEL, *Associate Editor*
Stanford University

H. ALBERT BARKER, *Associate Editor*
University of California

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PREFACE

The reception accorded the first volume of the *Review* has been so cordial that once again the editors and Editoral Committee wish to acknowledge their indebtedness to the reviewers whose superior efforts largely insured such a reception. The reviewers who made possible the second volume were faced with limitations of space, together with a wealth of material worthy of review. This made the task of compiling critical and, at the same time, comprehensive reviews a difficult one. These initial reviews in the various major fields of microbiology perhaps will lighten somewhat the task of future reviewers and enable them to present more critical analyses of current work.

The field of microbiology is so broad, and the interests in it so varied, that it is difficult to select topics for review in such a manner as to cover completely the various fields as they develop. To all who have made suggestions and criticisms in the planning of future volumes the committee expresses its appreciation. Our thanks are also extended to all who have provided reprints of their work for the use of the editors and the reviewers.

We wish to extend our thanks to Dr. Gunsalus and the *Annual Review of Biochemistry* for permission to reprint the review on Bacterial Metabolism [Ann. Rev. Biochem., **17**, 627–56 (1948)]. This topic will be incorporated in future volumes of the *Annual Review of Microbiology*. To the George Banta Publishing Co., and the office staff of Annual Reviews, Inc., we also express our gratitude for their hearty co-operation.

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H.A.B.
S.R.
C.E.C.

CONTENTS

YEASTS

By E. M. Mrak and H. J. Phaff[1]
Division of Food Technology, University of California, Berkeley, California

Introduction

The subject "yeasts" is very wide and many different aspects may be considered. Discussion herein, however, will be limited to their phylogeny, morphology, taxonomy, and ecology. The physiology of yeasts has been covered in recent reviews by Joslyn (67), Neuberg (134), Brandt (18), and Nickerson (136, 139). Stephany & von Loesecke (195) have compiled an extensive list of selected references of particular interest to the manufacturer of yeasts.

"Yeasts" are difficult to delimit, but they are commonly considered to include the ascospore forming organisms in the family *Endomycetaceae* as defined by Stelling-Dekker (194) and the imperfect genera included in the family *Torulopsidaceae*, which are discussed in the monographs by Lodder (106) and Diddens & Lodder (35). Such a limitation, however, is hardly acceptable from either a phylogenetic or taxonomic point of view. The contributions of Windisch (219, 220), Roberts (167), and Varitchak (197) show that organisms such as *Taphrina deformans* and *Ascoidea rubescens* are so closely related to the family *Endomycetaceae* that their inclusion in a discussion on yeast is well justified. Furthermore, when identifying yeasts it is difficult to exclude such commonly encountered genera as *Pullularia*, *Geotrichum* (*Oospora* or *Oidium* sometimes used as synonyms), or *Sporobolomyces*, none of which are given taxonomic consideration in the monographs cited above.

Terms such as cultured, true, false, wild, top, and bottom yeasts, used to define yeasts according to their industrial behavior [Laufer & Schwarz 90)], have been shown to have very little meaning. Winge & Laustsen (227) have observed intermediate forms between top and bottom yeasts and found that the ability to ferment one third or all of the raffinose molecule is not a reliable character to use in distinguishing these two types. Furthermore, while comparing wild and cultivated yeasts, they were unable to detect any fundamental morphological differences between the two types.

[1] This review covers the period up to January, 1948.

PHYLOGENY

Three views prevail concerning the phylogeny of yeasts: (*a*) They constitute a group of primitive *Ascomycetes;* (*b*) They are degraded forms originating in the *Euascomycetes;* (*c*) They are a mixture of primitive and degraded forms of polyphyletic origin.

Primitive origin.—The close relationship of the ascospore forming yeasts to the primitive *Hemiascomycetes* is a theory of long standing. Guilliermond (49) established the close relationship between the mycelium producing yeasts of the genus *Endomyces* and the budding forms, which were then grouped in the *Saccharomycetaceae.* This idea was further developed by Stelling-Dekker (194) and Kluyver (70), who revised the definition of the family *Endomycetaceae* to include the family *Saccharomycetaceae*; a logical change, for the limits of the two families were no means sharp. Stelling-Dekker also changed and expanded on the lines of development proposed by Guilliermond (50) and Gäumann & Dodge (42). Her system involves four lines of development through four subfamilies (see Fig. 1). The genus *Endomyces* as recognized by

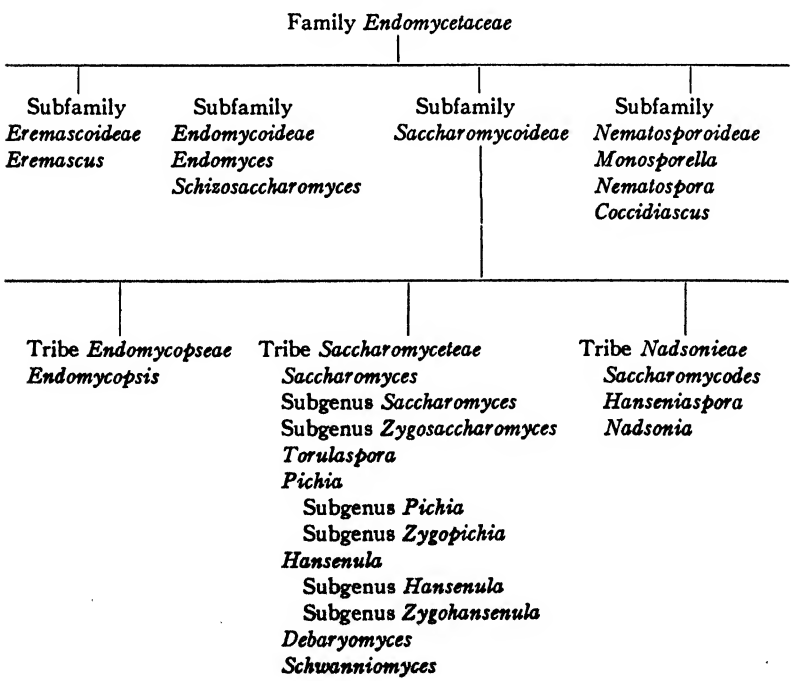


FIG. 1

Guilliermond (50) was divided into two homogeneous genera: *Endomyces*, characterized by the presence of arthrospores[2] and absence of blastospores[2], and *Endomycopsis*, producing blastospores only. A separate line of development then procedes from the genus *Endomyces* to the genus *Schizosaccharomyces*. The genus *Endomycopsis*, on the other hand, in view of its ability to produce blastospores, has been included in the tribe *Endomycopseae* of the subfamily *Saccharomycoideae*. This change avoids the confusing division of the genus *Endomyces* into two quite distant lines of development. The system proposed by Stelling-Dekker is undoubtedly the nearest approach to an inclusive natural system yet postulated for the yeasts.

Henrici (60) indicated that the relationship between the *Saccharomycetaceae* and *Endomycetaceae* is so obvious that it is now accepted by practically all mycologists. Some prefer to retain the two families as separate units, while others, including the authors, prefer to follow Stelling-Dekker and retain only the family *Endomycetaceae*.

The position of the family *Endomycetaceae* with respect to the *Phycomycetes* and higher *Ascomycetes* is becoming clearer. As early as 1915 Atkinson (4) pointed out that under certain conditions the asci of *Dipodascus* may be so reduced that they resemble *Eremascus* in number of nuclear divisions and ascospores. He indicated a possible line of development from *Dipodascus* through *Eremascus* and *Endomyces* to the less complex yeasts. In 1937 Biggs (14) described *D. uninucleatus*, which has characteristics that suggest a much closer relationship of *Dipodascus* to the *Endomycetaceae* than had been previously realized. The vegetative cells of *D. uninucleatus* are uninucleate whereas those of *D. albidus* are consistently multinucleate. Furthermore, under certain conditions of growth the thallus becomes yeast-like and the asci small and reminiscent of the *Endomycetaceae*. *Ascoidea* also appears related to *Dipodascus* and the *Endomycetaceae*. Gäumann & Dodge (42) included the families *Dipodascaceae*, *Endomycetaceae* (including *Ascoidea* and *Pericystis*), and *Saccharomycetaceae* in the order *Endomycetales*. Wolf & Wolf (230) have used a system employing the family name *Ascoideaceae* rather than *Dipodascaceae* to include those *Ascomycetes* forming plurinucleate gametangea and multispored asci.

[2] For definition of terms used in connection with the taxonomy of yeast see (88).

In addition to *Ascoidea* and *Dipodascus* they have included the interesting genus *Spermophthora* in this family. The genera *Ashbya* and *Eremothecium*, which Stelling-Dekker (194) excluded from the family *Endomycetaceae* because of the multinucleate condition, probably should be placed in the family *Dipodascaceae*. Skinner, Emmons & Tsuchiya (188) also used a system similar to that described above, except that the family *Endomycetaceae* included the *Saccharomycetaceae* as was suggested by Stelling-Dekker.

The concept of the close relationship between the *Endomycetaceae* of Stelling-Dekker and the *Ascoideaceae* of Wolf & Wolf obtains strong support from the morphological and cytological observations of Biggs (14) on *Dipodascus*, Walker (204, 205) and Varitchak (196, 197) on *Ascoidea* and *Pericystis*, and Guilliermond (56) on *Spermophthora*. This relationship now appears to be well enough established to warrant inclusion of these organisms in future taxonomic or phylogenetic considerations of yeasts.

Phycomycete characters, such as coenocytic cells and plurinucleate gametagnia, justify placing the family *Ascoideaceae* in the more primitive position between the *Phycomycetes* and the *Endomycetaceae*. This establishes the position of the yeasts between the *Phycomycetes* and the higher *Ascomycetes*. It is interesting to note that Mez & Ziegenspeck (123) were in accord with this when, by using their sero-diagnostic approach, they placed *Saccharomyces* between the *Zygomycetes* and the *Aspergillales*.

The more advanced, or highly developed, close relations of the yeasts are probably in the *Taphrinales*. Wieben (218), Windisch (219, 220) and Roberts (167) have established the close relationship of *Torulopsis pulcherrima* and *Taphrina deformans*. Roberts showed a great similarity between *Taphrina deformans* and *Torulopsis pulcherrima* in culture and was of the opinion that her cultural investigations lent support to the idea of general relationship between yeasts and the *Taphrinales*. Because of the absence of conclusive cytological data however, Roberts was not prepared to accept Windisch's proposal to transfer *T. pulcherrima* to a new family in the *Taphrinales*. Nevertheless, the observations of these investigators with respect to the occurrence of ascospore formation in *T. pulcherrima* and *Candida tropicalis* and their close relationship to *Taphrina* are very convincing. Further research on these organisms is greatly needed, for it is evident that the *Taphrinales* now require phylogenetic and taxonomic consideration with the yeasts.

Byssochlamys fulva is an unusual organism considered by Olliver & Smith (147) as a transition form between the *Endomycetaceae* and *Gymnoascaceae* in the *Plectascales* of the *Euascomycetes*. It produces conidia in chains on single or whorls of sterigmata, clusters of asci without a trace of peridium or enveloping hyphae and is capable of growing under reduced oxygen tension in canned fruits [Olliver & Rendle (146)]. Clements & Shear (30) included *Byssochlamys* in the family *Endomycetaceae*. Further study of the cytological characteristics of this little known genus is required for verification of its phylogenetic position. Other genera that have been assigned positions close to the yeasts are *Coccidioides*, by Moore (124), and *Rhinosporidium* and *Histoplasma*, by Wolf & Wolf (230). *Coccidioides* is probably closely related to *Protomyces* as pointed out by Baker, Mrak & Smith (6), but the position of *Protomyces* is uncertain. Gäumann & Dodge (42) place it in the *Hemiascomycetes*, whereas Fitzpatrick (41) feels that not sufficient is known about it to give it a definite taxonomic position. Skinner, Emmons & Tsuchiya (188) include *Coccidioides* in the *Zygomycetes*, but withhold designation of *Histoplasma*.

Degraded origin.—Bessey (13) considers the yeasts as reduced forms arising from certain genera in the *Euasomycetes*. Such a view has received little support from mycologists, for this system offers many more difficulties than that of the primitive origin.

Polyphyletic origin.—The view that yeasts represent a heterogeneous group of organisms derived from several different sources has received support from Zender (231), Krassilnikov (75), Guilliermond (55, 57), and Wallerstein & Schade (207). Zender based his concept primarily on the great differences in spore shape occurring in various genera and species. Krassilnikov also emphasized the importance of spore shape and postulated the scheme of development based largely on this characteristic (see page 6).

Krassilnikov expressed the opinion that Guilliermond, in formulating his early concept of phylogeny, stressed unduly the characteristics of the sex process and development but neglected ascospore shape. As an example it was difficult for Krassilnikov to conceive of *Endomyces decipiens*, a fungus with hat shaped spores, originating from *E. magnusii*, which produces spherical spores. Although the system proposed by Krassilnikov does not appear compatible with the basis on which it was developed, the principle of his scheme may have definite merit and should be given consideration in future modifications of phylogenetic theories. In fact,

ASCOMYCETES

Endomyces		Ascomycete B		Ascomycete A	
Eremascus				Saccharomycetes	
E. javanensis	*E. fibuliger*	*Guilliermondella selenospora*†	*E. magnusii*	*Saccharomycodes ludwigii*	*Zygosaccharomyces barkeri*
E. capsularis	*Hansenula*	*Guilliermondella sp.*‡	*Schizosaccharomyces octosporus*	*Nadsonia*	*Z. nadsonii*
E. schoenii	*Zygopichia*	*Saccharomyces marxianus*	*S. pombe*		*Debaromyces*
*Schwanniomyces Willia saturnus**	*Pichia*				*Torulaspora*

* *Hansenula saturnus.*
† *Endomycopsis selenospora.*
‡ Identity uncertain.

FIG. 2

his theory has already been applied by Stelling-Dekker in the grouping of genera with spindle shaped spores in the *Nematosporoideae.*

During the late years of his life Guilliermond (55, 57) revised his views concerning the development and relations of ascosporogenous yeasts. This revision was undoubtedly influenced by the observation of haploid and diploid generations in *Saccharomyces* by Satava (177, 178), Winge (221), and Winge & Laustsen (225, 226). He postulated two lines of development depending on whether the vegetative cells were haploid or dipliod. Yeasts with haploid vegetative cells were considered derived from the genus *Endomyces* and those with diploid vegetative cells from the genus *Taphrina.* Genera considered as derived from *Endomyces* were *Schizosaccharomyces, Zygosaccharomyces, Zygopichia, Debaryomyces, Nadsonia, Nematospora* (and presumably *Zygohansenula*). Those having diploid vegetative cells and derived from *Taphrina* were: *Saccharomyces, Hansenula, Pichia,* and *Saccharomycodes.* Guilliermond based his concept, to a large extent, on a series of contributions from his laboratory [Guilliermond (52 to 55), Manuel (113 to 117), and Renaud (159 to 163)]. Some of these certainly require verification before general acceptance. Specifically, it is questionable if one species of *Pichia* should be termed parthenogenetic while another shows conjugation of ascospores, or whether a dicaryotic condition has actually been seen in *Saccharomyces.*

Wallerstein & Schade (207) also suggested a system of development similar to that proposed by Guilliermond. Such a concept of the phylogeny of yeast cannot stand in the light of present day knowledge. It postulates that the two genera *Saccharomyces* and *Zygosaccharomyces* are but distantly related. Recent information indicates that these two genera should be combined into the single genus *Saccharomyces.* In 1931 Stelling-Dekker broadened the limits of *Saccharomyces* to include two subgenera, *Saccharomyces* (Sensu Strictu) and *Zygosaccharomyces.* Since then Winge & Laustsen (227) hybridized certain species of *Saccharomyces* and *Zygosaccharomyces,* thus demonstrating the close relationship of the two organisms. More recently Lodder (108) has shown that *Saccharomyces macedoniensis* and *Z. marxianus* are similar except for the point in the life cycle at which diploidization occurs. By use of

single spore isolations it was possible to transform one of these organisms into the other and vice versa. Such evidence leaves no basis for the polyphyletic scheme outlined above.

Basidiomycete origin.—Guilliermond (57) speculated on a possible relationship between yeasts and the *Ustilaginales*. It was suggested that the

> Chlamydospores of the *Ustilaginales* fulfill the functions of asci and may be considered homologues to the asci of *Exoascales*, for they first show two nuclei which later fuse through caryogamy.

This is followed by meiosis and the formation of a promycelium and basidiospores. Specific comparisons are made between diplobiontic smuts and yeasts such as *Ustilago carbo* and *Saccharomycodes ludwigii*, strains in which the haploid phase is restricted to a few cells such as in *U. violaceum* and *Saccharomyces*, and between smuts and yeasts which are definitely haplobiontic as *U. maydis* and *Zygosaccharomyces*. Though interesting, there is little basis for such comparison since Lodder (108) has shown that haplobiontic and diplobiontic organisms may merely represent two phases in the life cycle of a single species.

There is sounder evidence to indicate that the genera *Sporobolomyces* and *Bullera* are *Basidiomycetes*, although this has been a very controversial point. Kluyver & van Niel (72, 73), Derx (33), Buller (19), Henrici (60), and Langeron (88) support the hypothesis of affinity with the *Basidiomycetes;* whereas Lohwag (109), Guilliermond (51), and Stampell (191) consider such a view untenable. Ciferri & Verona (28, 199) indicate that our present knowledge concerning these organisms does not justify any conclusion. The thorough studies of Buller (19) are quite convincing to the writers. He considers the *Sporobolomyces* of hymenomycetous origin and that the mycelium has become adapted to the habit of budding (characteristic of an aqueous environment); the original fruiting body with four spored basidia has been lost and the original mode of development and discharge of basidiospores has survived. Possible reasons given for the absence of conjugate nuclei are the loss of sex or the existence of heterothallic strains when haploid cells produce two spores and diploid cells four spores. The mode of development and liberation of spores is considered an important character.

Anascosporogenous yeasts.—This group presumably has arisen primarily from ascosporogenous yeasts losing their ability to sporulate. Such a change was first observed by Hansen [see Lafar (86)]

in *Saccharomycodes ludwigii* and by Beijerinck (10) in *Schizosaccharomyces.*

In other instances the loss of sporulation may occur when single ascospores germinate without mating with their complementray type. Such a culture may either remain haploid or become diploid, by the fusion of sister cells, to form the so-called illegitimate diploid. Such diploid cultures frequently lose their ability to sporulate, and if spores are formed they are generally of low viability. This behavior was first observed by Kruis & Satava (79) and Satava (178) and confirmed by Winge & Laustsen (225, 229) and Lindegren (92).

Diddens & Lodder (34) pointed out the relationship between the following perfect and imperfect yeasts: *Saccharomyces fragilis* and *Candida pseudotropicalis*, *S. macedoniensis* and *C. macedoniensis*, and *Hansenula javanicus* and *C. pelliculosa.* Laffer (87) reported a throat infection first presumed to be caused by *Candida pseudotropicalis* which later, upon observation of ascospores, was found to be *Saccharomyces fragilis.*

Torulopsis pulcherrima and *Candida tropicalis* appear to represent life phases of certain Taphrinales (219, 220). Baltatu (7) maintains that species of *Mycoderma* are sporulating yeasts very similar to *Pichia*; these observations, however, have not been confirmed by others.

Lodder (106) and Henrici (60) have indicated that *Sporobolomyces* maintained in a culture irreversibly lose their power to form basidiospores and become indistinguishable from the ordinary red yeasts of the family *Rhodotorulaceae.* It cannot be assumed from this alone that all *Rhodotorula* isolates represents imperfect stages of *Sporobolomyces.* Mackinney & Mrak (111) have compared the absorption spectra of the pigments of a limited number of cultures of the two genera and found them to be quite different. Further information of this type would be helpful in establishing the possible affinities of the two genera.

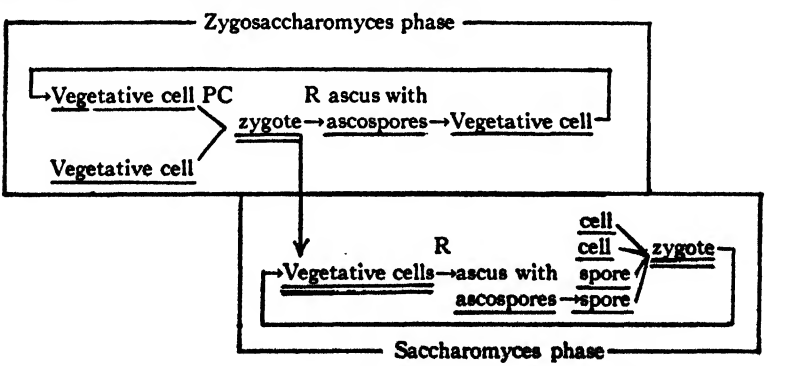
Haploid and Diploid Generations

Phaff & Mrak (153) have reviewed the literature on sporulation in yeasts and discussed the various types of life cycles. The occurrence of two generations in such yeasts as *Schizosaccharomyces*, *Zygosaccharomyces*, *Zygopichia*, and *Debaryomyces*, in which the zygote represents the diploid, and ascospores and vegetative cells the haploid phase, has been accepted for a long time. *Sac-*

charomyces and a number of other genera, however, were generally considered parthenogenetic until Satava & Kruis (79, 177, 178) and Winge (221) showed the existence of two generations. The ascospores either fuse or form small haploid cells by budding which may fuse in pairs or with a spore to form the diploid zygote. The latter, however, develops large vegetative cells rather than ascospores as in *Zygosaccharomyces*. Hence, *Saccharomyces* has a diploid phase represented by vegetative cells and the haploid by ascospores. According to Manuel (116), certain species of *Hansenula* and *Pichia* behave in a similar manner. Presumably the nuclei fuse upon anastomosis of the cell [Gäumann & Dodge (42)], although Guilliermond (57) and Renaud (163) presented slight evidence to show the occurrence of a dicaryotic stage.

The principal difference in the life cycle between *Saccharomyces* and *Zygosaccharomyces* is the number of vegetative generations, which the organism spends in the haploid or diploid phase. That the subgenera are very closely related has been well established by contributions of Winge & Laustsen (227) and Lindegren (92) showing that intergeneric hybridizations may be accomplished between certain strains of the two subgenera.

Lodder (108), in addition, showed that *Saccharomyces macedoniensis* represents the diplophase and *Z. marxianus* the haplophase of the same species. She considers the evidence sufficient to show that *Zygosaccharomyces* does not deserve subgeneric rank and *Saccharomyces* should supercede the two existing subgenera. Phaff & Mrak (153) diagrammed the life cycle of the genus *Saccharomyces* embodying the two subgenera as follows:[3]

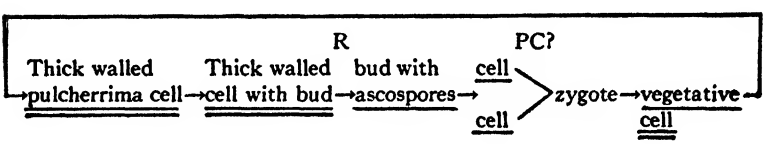


[3] P, refers to plasmogamy, C, to caryogamy, R, to reduction division, the single lines, haplophase and the double lines, diplophase.

In view of the observations of Manuel (113, 116) it is logical to believe that such a life cycle would also apply to *Pichia* and *Zygopichia*, and *Hansenula* and *Zygohansenula*. Manuel maintained that *P. mandshurica* is parthenogenetic. It appears more likely that nuclear fusion takes place between the mother-nucleus and the daughter-nucleus immediately at the germination of the spore, similar to that in *Saccharomyces* as pointed out by Winge & Laustsen (225).

Manuel (115) and Guilliermond (57) state that isogamic conjugation occurs in *Nematospora coryli* and the ascus results from the complete fusion of two gametes. This is a surprising statement for neither Stelling-Dekker (194) nor the writers have seen conjugation in this organism.

On the basis of information presented by Windisch (219, 220) and Roberts (167), Phaff & Mrak (153) have diagrammed the life cycle (in culture) of *Torulopsis pulcherrima* (*Taphrina*) as follows:



Genetics

The subject of yeast genetics was reviewed by Winge (222) and Lindegren (92). Two subsequent papers by the Lindegrens (93, 97) have been published in support of their theory pertaining to the existence of a cytogene and its behavior during the presence and absence of a particular substrate available to the cell. In the discussion following one of the papers (97) given at a Cold Spring Harbor Symposium, Sonneborn voiced a strong skepticism of the cytogene and pointed out that it "calls for relentless criticism and the most unobjectionable experiments until it is securely established or discarded."[4]

Contributions by Lindegren & Lindegren (95) and Skoog & Lindegren (189) have been concerned with adaption and the relation of the composition of the substrate to the appearance of "mutants" in yeasts. Lindegren & Raut (99, 100) showed that

[4] Winee & Roberts in a recent paper [*Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **24**, 263–315 (1948)] also rejected the cytogene theory.

strains of *S. cerevisiae*, which normally cannot synthesize pantothenic acid, may undergo mutation and become able to synthesize this vitamin when the level of pantothenate in the medium drops below a certain minimum; but some panthothenate (either in the cell or in the medium) must apparently be present before the synthesizing mechanism can operate. They suggested that these changes might be cytogene additions, since they are quite different from the ordinary recessive mutations used in genetic Mendelian analysis. Lindegren & Lindegren (95) have also presented evidence to show that genes affecting vitamin synthesis in yeasts may segregate in regular Mendelian manner in selected inbred pedigrees. Also of interest is the observation of a depletion mutation by Lindegren & Lindegren (96) in which methionine is required for the development of a pink color in a mustard gas treated haplophase segregant of *S. cerevisiae*. A hybrid heterozygous for adenine-dependence and methionine-dependence was produced. Of the segregants, the adenine-dependent white cultures were also methionine-dependent and all the adenine-dependent pink cultures were methionine-independent, indicating that methionine was required for development of the pink color. It was pointed out that false "mutations" from pink to white may appear in adenine-dependent, methionine-dependent cultures when growth occurs in the absence of sufficient methionine to insure color production. Color variation has also been studied in *Torulopsis pulcherrima* by Punkari & Henrici (155, 156), who found a marked dissociation of colonies, arising from single cells, into red and white areas; the white colonies showing greater stability than the red. Lodder (106) and Castelli (24) indicated that the presence of iron in the medium is essential for color formation; whereas Porchet (154) obtained no color change by adding iron. Roberts (166) carefully studied the effect of temperature, oxygen, light, amount of substrate size and position of the colonies, and iron on color production. The pigment intensity increased gradually with increasing concentration of ferric ammonium sulfate up to 0.1 per cent in the medium. Furthermore, a relationship was found between pigmentation and the total amount of nutrient substances available to the colony. In the absence of oxygen pigment formation was inhibited.

Nadsonia richteri is notable for color sector formation in giant colonies. Skovsted (190) observed this sectoring to occur through

several successive generations even when single isolates were used. Each type under fixed conditions produced a limited number of mutants, and the mutants in turn produced new mutants and so on. In some cases the variations were so great that they might well have been considered as new species. Back mutations were not observed. For this process, Skovsted proposed the term "Successive Mutations."

Winge & Laustsen (228) found *Saccharomycodes ludwigii* to be a balanced double heterozygote of the genetic formula NnLl (normal and abnormal and long and short cell growth). They studied the germination of single spores of *Saccharomycodes* and observed that two of the four spores in an ascus germinate normally and produce unlimited growth of haploid cells, whereas the two others germinate with an elongated mycelial cell producing at most one or two transverse walls before they definitely cease growing and die. Furthermore, the two normally germinating spores in an ascus either germinate both with short cell growth or both with long-cell growth. When the spores once produce haploid cells fusion cannot take place between these cells, and the ability to sporulate is lost. A further explanation of this behavior was given recently by Winge (224), who pointed out that after a prereduction in which L is continually separated from l and N from n the nuclear spindles of the two nuclei arrange themselves parallel with the longitudinal axis of the ascus, so that both nuclei give off a daughter nucleus to each end of the ascus. Winge found certain asci having the genetic formula NNLl in which the lethal gene was absent. In another publication Winge (223) criticized the work of Manuel (117), who claimed to have produced giant colonies from the abnormal (n) single ascospores of *Saccharomycodes* asci. Ditlevsen (36) also found *Saccharomyces italicus* to be heterozygotic for a gene concerning cell form. When the four spores of an ascus were isolated and grown separately, two formed short-cell colonies and two formed long-cell colonies.

Methods of Study

The identification of yeasts requires the use of both morphological and physiological characters. The procedures used in determining these characteristics are given in the forementioned three monographs on yeasts (35, 106, 194). A more inclusive, though

seldom used system, has been suggested by Perotti & Verona (151). These methods have limitations and in certain instances improvements have been suggested.

Isolation.—The separation of a yeast from a mixed culture with mold has always been a difficult problem. Hertz & Levine (62) successfully employed diphenyl (100 p.p.m.) in nutrient and malt agar to recover yeast cultures which had become contaminated with mold. The use of diphenyl, however, is inconvenient because of its insolubility and volatility, which results in loss of fungistatic activity on prolonged incubation. Sodium propionate in concentrations of 1, 2, and 3,000 p.p.m. in malt extract agar at pH 4.7 was also tried with limited success. At a concentration of 3,000 p.p.m. all molds were inhibited, but growth of many of the yeasts was also markedly retarded. At 2,000 p.p.m. the propionate was less detrimental to the yeast, but inhibition of mold growth was insufficient to warrant its use. The writers, on the other hand, have found a 5 per cent wort agar containing 2,500 p.p.m. sodium propionate quite useful in separating yeasts and molds. Effectiveness of the propionate increases when the pH is lowered [Bornstein (15)]. The pH of the medium used in our laboratory is approximately 5. Since the growth of yeast is also retarded by the propionate (although less so than the molds) it may be necessary to increase the incubation period by a few days.

Pityrosporum ovale can be isolated easily by planting scales from Seborrhea oleosa (dandruff) in an acid dextrose broth containing 23 to 44 per cent glycerol and incubating between 30° and 37°C. [Emmons (38)]. The important function of the glycerol is to inhibit the growth of contaminants. Some of the growth requirements of the yeast are contained, apparently, in the scales used in the inoculum. For subsequent culturing, however, it is essential to add lanolin, oleic acid, butter, or similar substance to the surface of the medium. Benham (12) described a simple technique for accomplishing this, by pipetting an ether extract of crude lanolin or butter over the surface of the agar and then allowing the ether to evaporate.

Ascospore formation.—In their review on the sporulation of yeasts Phaff & Mrak (153) discussed factors involved in production of ascospores and have given the important procedures and

media used to induce ascospore formation. The cells must be young and active when transferred to the sporulation medium. In order to secure this condition Lindegren & Lindegren (94) used a presporulation medium containing fruit and vegetable extracts, brewers' yeast, glycerol, and calcium carbonate. Baltatu (7), on the other hand, used plain grape juice, but passed the yeasts through this medium a number of times before transfer to a sporulation medium.

A vegetable extract (beet, carrot, cucumber, potato) agar formulated by Mrak, Phaff & Douglas (129) has proven to be an effective sporulation medium. Henrici [cited in (217)] simplified preparation of this medium by using a commercially available blend of eight vegetable juices and agar. By including bakers' yeast Wickerham *et al.* (217) further increased its effectiveness. It has also been our experience that inclusion of dried brewer's yeast in the medium of Mrak *et al.* (129) increases its effectiveness considerably.

The influence of metabolic products of microorganisms on sporulation has recently received the attention of a number of investigators. Windisch (219) reported that heterogamic conjugation of *Torulopsis pulcherrima* was strongly stimulated when grown with a culture of *Penicillium* in a droplet of wort. Nickerson & Thimann (141, 142) were able to concentrate and partially identify the substances produced by *Aspergillus niger*, capable of stimulating the conjugation of vegetative cells in several species of *Zygosaccharomyces*. The active substances were presumably identical or related to riboflavin and glutaric acid. However, the activity of this mixture was less than that of the filtrate of *A. niger*, containing a similar amount of the active substances, indicating that the fungus filtrate contained additional stimulatory compounds. While observing cross sections of yeast colonies, Lindegren & Hamilton (98) noted that the outer layer of autolyzed cells was the only region in which sporulation occurred. This suggests that, besides the possible effect of oxygen, the autolysate may supply substances essential for sporulation. The writers have obtained an abundance of sporulating cells of *Saccharomyces cerevisiae* and *S. carlsbergensis* by streaking young cultures on agar slants prepared with 20 volume per cent of yeast autolysate in tap water containing 0.1 per cent mono-potassium phosphate and 0.05 per cent of magnesium sulfate. The study of specific chemical stimu-

lation of sporulation certainly merits further consideration. For a more inclusive discussion on the effect of other chemicals on sporulation see Phaff & Mrak (153).

Fermentation.—Kluyver (70) made three generalizations concerning the fermentation characters of yeasts: (*a*) a yeast will not ferment any other sugar if it does not ferment glucose; (*b*) all yeasts which ferment glucose also ferment fructose and mannose; (*c*) a yeast is never capable of fermenting both maltose and lactose. With but one exception, these generalizations are still correct. Custers (32) pointed out that Schiönning (180) had described a yeast, termed *Torula* B, capable of fermenting both maltose and lactose. Custers confirmed this observation and renamed this organism *Brettanomyces claussenii.* Sacchetti (174) and Bouthilet *et al.* (17) have observed an apparent fermentation of both maltose and lactose in a limited number of yeasts, and later showed that a fermentation of comparable strength was also observed in plain yeast extract without any added sugar. Bouthilet *et al.* (17) demonstrated that the material responsible for this "blank" fermentation and the apparent fermentation of lactose was the dissacharide trehalose which is one of the reserve carbohydrates of bakers' yeast. Trehalose is destroyed by autolysis, so a 10 or 20 per cent autolysate rather than yeast extract is recommended as the basic fermentation medium, free of fermentable carbohydrates. A number of yeasts were observed to ferment trehalose, and in one case the rate of fermentation was increased by first growing the cells in the presence of trehalose. The fermentation of trehalose was always apparent in Durham tubes but in several cases was not apparent in Einhorn tubes, even after shaking. This indicates the greater sensitivity of Durham tubes as compared to the Einhorn tubes, which were used by Stelling-Dekker.

The fermentation of melibiose is of particular importance in distinguishing certain species of *Saccharomyces.* Melibiose as such is seldom used, however, because of its high cost; raffinose being used instead. This has required the use of a quantitative fermentometer, such as the van Iterson-Kluyver tube, to determine whether one-third (fructose only) or the entire molecule (fructose and melibiose) is fermented. Wickerham (213) outlined a simple technique involving the use of a 4 per cent raffinose solution in Durham tubes. The tube is inoculated with the test culture and allowed to stand until fermentation has passed its maximum as judged by

gas collection in the vial. When gas in the insert begins to decrease the tube is again inoculated with a culture of *S. carlsbergensis*, which is capable of fermenting melibiose. If additional gas is produced the yeast under test failed to ferment melibiose. On the other hand, if no gas is produced the yeast under test is considered a melibiose fermenter. Skinner & Bouthilet (187) modified this procedure by fermenting the fructose portion of raffinose in 3 per cent raffinose broth with *S. cerevisiae* var. *ellipsoideus* prior to tubing and then resterilizing the fermented broth.

Auxanograms.—Beijerinck's auxanographic method for testing the utilization of certain compounds for growth was applied to yeasts by Lodder (106). Nitrogenous substances or carbon sources can be tested in agar media containing suspensions of yeast. The medium is so composed that it is deficient in one of these two groups of essential nutrients. A small quantity of the substance to be tested is placed on one small area of the plate. At least five substances can be tested on each plate. The plates are then incubated and observed for opaqueness, which indicates utilization and growth. Wickerham (215) found that the bios requirement in these synthetic agar media (which Lodder considered satisfied by using a heavy inoculum of the test organism) was not fulfilled in the case of certain yeasts, especially the apiculate forms. According to Lodder these organisms only use peptone of the five nitrogen sources tested by her. Wickerham showed that they will also utilize ammonium sulfate, urea, and peptone if the medium is supplied with a mixture of B-vitamins. Nitrate was not utilized in spite of the addition of these vitamins. Custers (32) showed that, besides nitrate, nitrite is also utilized as a nitrogen source by species of *Brettanomyces*, but nitrite becomes more toxic when the pH is lowered and therefore should be tested at about neutrality. Because of the slow growth of *Brettanomyces* species, Custers applied the various sugars on a sugar auxanogram plate not in the form of crystals but as a droplet of a sterilized concentrated solution in distilled water. Nickerson (137) used a liquid medium for testing nitrate utilization and was able to show the presence of nitrite as an intermediate in cultures of *Hansenula*, utilizing nitrate, but not in *Pichia*, which cannot use nitrate.

Kluyver & Custers (71) showed that some yeasts which can ferment glucose, fructose and mannose, but not certain disaccharides such as sucrose and lactose, may show a positive sugar auxano-

gram for these sugars. They reject the idea of direct respiration of these disaccharides (that is without previous hydrolysis) but were able to demonstrate a weak hydrolase enzyme in such yeasts, which might be for the most part inactive under anaerobic conditions, thereby, accounting for negative fermentation tests of the forementioned disaccharides.

Carotenoid pigments.—The families *Rhodotorulaceae* and *Torulopsidaceae* are distinguished by the occurrence of carotenoid pigments in the former and not in the latter. Lodder (106) used the alcoholic potassium hydroxide method of Molisch to demonstrate the presence of these pigments, apart from visual observation. The writers have had uniformly negative results with this procedure. Mackinney (110) found that several species of *Rhodotorula* and *Sporobolomyces* resist boiling in alcoholic potassium hydroxide for several minutes without liberation of the pigments. The pigment was extracted, however, by careful hydrolysis with concentrated hydrochloric acid diluted 1:1 and subsequent extraction with acetone, followed by petroleum ether. On the basis of this the following satisfactory procedure for the demonstration of carotenoid pigments in yeasts has been developed by the authors. The yeast growth is scraped from a heavily inoculated plate and mixed thoroughly with about 10 ml. of water in a large test tube. Ten milliliters of concentrated hydrochloric acid is added and the mixture heated to a boil. The tube is cooled immediately in cold water. When cool, 50 ml. of acetone are added and carefully shaken. A shallow layer of petroleum ether (one-fourth to one-half inch) is then added and shaken gently. Upon standing a few minutes the petroleum ether layer on top shows the dissolved pink pigment if the yeast contains carotenoid pigments. The writers have tried this method with success on about forty different cultures of *Rhodotorula.* The test shows negative results with pink or reddish cultures of *Torulopsis pulcherrima, Pichia,* and others.

Synthetic media.—Most media used in studying yeast are of uncertain composition. Bedford (9) used a synthetic medium while studying film formation and cell morphology of *Hansenula* and *Pichia.* His medium (0.1 per cent potassium dihydrogen phosphate, 0.1 per cent ammonium sulfate, .05 per cent magnesium sulfate, and 5 per cent glucose) has the advantage of reproducibility, but the limitation of being inadequate to support the growth of many yeasts aside from *Hansenula* and *Pichia.* Further devel-

opment of this medium to include sufficient growth factors to satisfy the requirements of all yeasts and its general use for morphology merits consideration, for it would reduce taxonomic discrepancies resulting from variations in media.

Colony characteristics.—Characteristics of giant colonies may vary greatly in certain organisms, for example, in *Nadsonia richteri* (190) and in *Eremothecium* [Arragon *et al.* (3)]. The variation may be so great that the interpretation of giant colony characteristics in species differentiation must be done with great care. Mackinnon (112) and Skinner (186) have carefully reviewed the extensive work on colony variations in *Candida albicans*. Of particular interest is the occurrence of S and R forms and the use of blood agar [Conant (31)] to separate S and R forms. Giant colonies have been very useful in establishing identical or dissimilar ascospores, separated from asci by micromanipulator technique [Winge (221, 222)]. They may show whether a yeast is homo- or heterozygous for certain characters. The authors have found giant colonies a useful guide, when large numbers of cultures are isolated from certain substrates, to indicate whether isolates are identical. We also have frequently found large numbers of ascospores in giant colonies, particularly in *Zygosaccharomyces* and *Hanseniaspora*.

Gelatin liquefaction.—Mrak, Phaff & Vaughn (131) have observed that sporulating cultures of an apiculate yeast (*Hanseniaspora*) liquefy gelatin when spores dehisce, apparently because of the liberation of protoplasm upon fragmentation of the asci. The nonsporulating apiculate yeast *Kloeckera*, on the other hand, was not observed to liquefy gelatin. Gelatin liquefaction does not appear to be a very reliable character, in our experience, for differentiation of yeasts.

Storage of cultures.—Henry (61) described a method for storage of yeast cultures under sterile mineral oil. The writers have used a similar procedure for nine years on more than 600 cultures representing all genera, except *Trigonopsis* and *Pityrosporum*, and have found it satisfactory except for cultures of *Brettanomyces*. These must be stored on chalk agar and transferred every six weeks. Cultures stored under oil should be transferred once a year, although we have found that many cultures remain viable for three years or longer. Some cultures of *Rhodotorula* have a tendency to lose color when held under oil.

Wickerham & Flickinger (216) preserved over one thousand

cultures for about two years by the lyophil process and about 98 per cent of the cultures were alive after this period.

Taxonomy

In addition to the monographs of Stelling-Dekker (194), Lodder (106), and Diddens & Lodder (35) a considerable literature on yeast taxonomy has appeared. Only the more important developments can be discussed here. In revising the genus *Hansenula*, Bedford (9) proposed the use of a new medium for studying the morphological characters of this genus and described a new species *H. subpelliculosa* which forms an extremely thin pellicle. This requires broadening of the genus definition. Mrak *et al.* (132) pointed out that the genera *Hansenula* and *Pichia* are now very similar except for the utilization of nitrate by *Hansenula* and not by *Pichia*. Nickerson (138) has redefined the subgenus *Zygopichia* and included in it film forming *Zygosaccharomyces*.

The key for the genus *Debaryomyces* of Stelling-Dekker (194) has been improved and made more usable by Lodder (105). The contributions of Diddens & Lodder (35), Mackinnon (112), and Skinner (186) have done much to clarify the confused taxonomy of *Candida*. Nevertheless, the basis for species differentiation is so broad that it is sometimes quite difficult to identify species. This is particularly true of *Candida albicans*.

Custers (32) prepared a monograph on the genus *Brettanomyces*, the so-called "ale" or "lambic" yeasts. He formulated a key which includes four species and two varieties. Two new species were described (*B. anomalus* and *B. claussenii*) and two new varieties (*B. bruxellensis* Kuff. et v. Laer var. *lentus* and *B. bruxellensis* Kuff. et v. Laer var. *non-membranaefaciens*). Custers placed the group as a separate genus in the *Mycotoruloideae* based on the presence of a pseudomycelium with a primitive blastospore apparatus, ogive cell shape, and aerobic acid volatile production. This classification was accepted by Diddens & Lodder (35), who included this genus in their monograph.

Diddens & Lodder (35) revised and prepared a key to the species of the genus *Trichosporon*, the third genus in the *Mycotoruloideae*. They described six species and one variety including two new species, *T. fermentans* and *T. capitatum*. They pointed out that in this genus physiological characters are important in species

differentiation, since morphological characters are very similar for most species and, moreover, are often quite variable within a certain species.

The genus *Asporomyces* of the *Torulopsidoideae* was tentatively retained by Lodder (106), but Mrak, Phaff & Smith (130) have shown that since the formation of abortive conjugation tubes is not a reliable character for generic differentiation the genus *Asporomyces* is no longer acceptable. In accordance with this view they revised the key to the genera of the subfamily *Torulopsidoideae*, omitting *Asporomyces*.

Ciferri & Verona (28) characterized the family *Nectaromycetaceae* (including the genera *Sporobolomyces*, *Nectaromyces*, and *Bulleria*) primarily on the basis of spore discharge and mirror image formation. *Sporobolomyces* produces conidia only on short simple sterigmata and may form pseudomycelium (subgenus *Blastoderma*) or it may be absent (subgenus *Sporobolomyces*). *Nectaromyces* has blastospores in tetrads ("aeroplane-like"), branched sterigmata, and pseudomycelium. *Bulleria* produces asymmetric conidia on simple sterigmata but no pseudomycelium. We feel, however, that there is little basis for this number of genera and the criteria used for their differentiation. Diddens & Lodder (35) logically included *Nectaromyces* in the genus *Candida*. It is very doubtful if *Blastoderma* is a valid genus, since Imshenezky (66) found it possible to propagate mycelial forms and non-mycelial forms of *Sporobolomyces* by transferring cells from various areas of the giant colony. The genus *Bulleria* is likewise of doubtful validity since asymmetric conidia and absence of color [Derx (33)] may result from variations occurring in the genus *Sporobolomyces* [Imshenezky (66)]. Verona & Ciferri (199) again revised the genus *Sporobolomyces* and included three new species. Optimum and maximum temperatures (given in some instances to 0.5°C.) are used as important characters of differentiation in the key. In our experience, temperatures at which growth occurs is not reproducible to 0.5°C. so if the key is used it must be done with reservation.

Because of the close resemblance of *Torulaspora rosei* to *Zygosaccharomyces globiformis* there has been some question of the validity of the genus *Torulaspora*. Krumbholz (82) raised the question and was supported by Sacchetti (175). Castelli (24), on

the other hand, claimed that ascospore formation in twenty cultures of *T. rosei* and one of *Z. globiformis* rather than *Torulaspora* is questionable. Nickerson (136) has pointed out, however, that it is not clear whether or not Castelli tried to duplicate Krumbholz's conditions. Since Lodder (108) has shown that *Zygosaccharomyces* is merely a haplophase of *Saccharomyces*, it is conceivable that *Torulaspora* may be another variation of *Saccharomyces*.

The controversial genus *Kloeckeraspora* has bipolar budding and one spherical spore per ascus according to Niehaus (143) and Dvornik (37). Castelli (20), on the other hand, maintains these structures are not ascospores because they fail to show germination, or exhibit greater heat resistance than the vegetative cells. This particular question warrants further investigation, but even if the spherical bodies prove to be ascospores, there is no reason for establishing a new genus on the basis of ascospore number and shape alone. A similar opinion was expressed by Henrici (60). A slight modification in the definition of *Hanseniaspora* would permit inclusion of "*Kloeckeraspora*" in that genus.

Some interesting species have been described since the appearance of the three Dutch monographs. Wickerham & Duprat (214) gave a complete description of *Schizosaccharomyces versatilis* nov. sp. It usually has eight ascospores, but its fermentation characters are quite different from those of *S. octosporus*. One interesting biochemical property is that it ferments melibiose, but not galactose. In other words, melibiose is fermented half and raffinose two-thirds. The nucleus appears frequently visible in the living cells, which is very unusual for yeasts. On solid media this *Schizosaccharomyces* tends to form hyphae which grow down into the agar. Hyphal growth might indicate its descent from the genus *Endomyces* or at least a very close relationship. An agglutinating form arises infrequently from a normal form of growth.

Holst (65) described *Z. pini* characterized by forming hat shaped ascospores. *Z. pastori* is the only other member of this genus forming hat shaped spores.

Nickerson (135) described *Zygossacharomyces acidifaciens* nov. sp. and Nickerson & Carroll (140) reported on some of its biochemical properties. It produces a high volatile acidity and will dissolve 0.5 per cent calcium carbonate in Custer's glucose yeast extract-calcium carbonate agar when grown under anaerobic conditions

(see section on Ecology). It appears to us that its taxonomic position is not very certain, since it assimilates nitrate, a character which, heretofore, has not been reported to occur in species of either *Saccharomyces* or *Zygosaccharomyces*. Even though no pellicle is formed it might fit in the genus *Zygohansenula* since Bedford's new species *H. subpelliculosa* is also practically devoid of a film. *Zygossaccharomyces* is one of the most difficult and confusing yeast genera and revision is badly needed. Combination of the two subgenera *Zygosaccharomyces* and *Saccharomyces* into the single genus *Saccharomyces* as suggested by Lodder (108) would make the task very difficult.

The so-called "Jerez yeasts" involved in the production of certain types of sherry wines are strongly fermentative organisms characterized by a delayed film formation. According to Schanderl (179) this organism has been termed *Saccharomyces cheresiensis* by Russian workers. He indicated, however, that species designation on the basis of delayed film formation is not justified since this character is common to a number of species of *Saccharomyces*. Hohl & Cruess (64) isolated representatives of *Pichia*, *Hansenula*, *Saccharomyces*, and *Torulopsis* from Chalon and Spanish wine films. After making an extensive study of these organisms in Spain, Marcilla, Alas & Feduchy (118) designated them as *Saccharomyces beticus* nov. sp. and listed two races. The new species is unusual in that it ferments only glucose, fructose, mannose, and sucrose. Film formation requires five to nine days. The other races, in our opinion, are more similar to *Saccharomyces chevalieri* or one of its varieties.

Kudriavtzev (84) claimed that *Saccharomyces cerevisiae* will ferment a trisaccharide contained in "beer wash," whereas *S. cerevisiae* var. *ellipsoideus* will not ferment this sugar. On the basis of this difference, and the fact that he was unable to find *S. cerevisiae* in nature Kudriavtzev recommends re-establishing the variety *ellipsodideus* as a distinct species. The use of environment as a species character, however, is hardly reliable. In contrast to Kudriavtzev we have repeatedly isolated a number of cultures of *S. cerevisiae* from fruit and grapes in California, but seldom cultures corresponding to Stelling-Dekker's definition of the variety *ellipsoideus*. Schultz, Atkin & Frey (183) proposed the subclassification of species into biochemical types on the basis of response to certain growth factors. This idea was later carefully worked out

by Schultz & Atkin (182) and applied to the biochemical classification of a number of *Saccharomyces* strains and isolates of other genera. They proposed a relatively simple method to determine the requirements of seven vitamins in chemically pure form and suggested naming of strains by their "bios number" (BN). Such a number corresponds with one or more of the seven vitamins for which the yeast is deficient. Examples and complete experimental details were given. The authors also offered the useful suggestion of including in addition to the bios number, by a system of letters behind the name of a yeast, the sugars which it is able to ferment.

The generic names, *Torulopsis* and *Crytococcus*, are both used for an imperfect group of yeasts. Lodder (107) has made it clear that the generic name *Cryptococcus* Kutzing should be avoided in yeast taxonomy and that *Torulopsis* is the valid name for this group of asporogenous yeasts. Benham (11), on the other hand, maintains that the generic name *Cryptococcus* has priority. The writers have concluded that priority is practically impossible to establish when one must rely on the original descriptions which are so very incomplete and which antedate, by many years, the use of pure cultures. In the end such a matter can only be settled by a committee on nomenclature.

There are many other contributions dealing with taxonomy; some of the more important ones are: Castelli (21, 22, 23), Conant (31), Ciferri & Verona (29), Kufferath (85), Langeron & Guerra (89), Martin *et al.* (120), Guilliermond (53), Puntoni (157), Sacchetti (171, 176), Scrivani (184), Verona (198), Verona & Luchetti (201), Verona & Valleggi (202), and Zimmermann (233).

Ecology

Numerous distribution studies have shown that yeasts are not as ubiquitous as bacteria, but display definite trends for certain habitats. Not only are yeasts found less frequently in nature than bacteria, but the different types of yeasts are very often found only on certain substrates of special chemical composition and concentration. It is unfortunate that yeasts described in many of the older publications are usually so poorly defined that their identity is, in most cases, highly uncertain. Certain yeast floras are found on substrates high in sugar and salt content, or those which contain special carbohydrates, such as lactose, starch, dextrins, wood,

etc. Others are particularly adapted to the utilization of special nitrogen sources such as nitrate; whereas others cannot use nitrate but utilize ammonium salts well, and still others seem to be dependent on proteinaceous materials. Unless special precautions are taken, ordinarily only the predominant organisms will be isolated by direct plating. It is more difficult to use selective media in the case of yeast than bacteria, because of less variation in metabolism. Yet the authors have had some success in isolating special yeasts by the use of selective media. Yeasts do not only occur as spoilage organisms, but some participate in special types of fermentations which will be discussed below. In surveying the literature the authors were impressed by the large number of new species and varieties described by various groups of workers. We feel that most of these "new" organisms are unjustly named for several reasons. The investigators often lack authentic cultures or carefully checked diagnoses to compare with their cultures; sometimes they are unfamiliar with the literature; and quite often the species concept is interpreted in an exaggerated sense. The Dutch monographers rejected many new species because they proved identical or at least very similar to previously described ones. Broadening of the species concept is highly recommendable in our opinion, so that slightly different organisms may be classified more easily. Varietal or strain differences may then be established depending on the purpose for which they are needed.

Yeasts occurring in substrates with a high sugar content.— Krumbholz (83) reviewed the literature relating to the spoilage of foodstuffs of high sugar content by yeast fermentation. Fermentation of stored honey by sugar tolerant yeasts has been studied extensively by several groups of workers, since it constitutes an important form of spoilage. It appears, according to several investigators, that in isolating yeasts from honey or other sources with high sugar concentrations best results are obtained by direct plating on media of high sugar content. When the syrups are plated on agar media of low sugar content or centrifuged and washed with sterile water and then plated on high Brix degree agar plates, usually no growth occurs. Lochhead & Heron (103) found it necessary to use 50° Brix honey agar to obtain proper colony development, lower sugar concentrations giving poor or no growth. In our opinion the semisynthetic honey media employed

may have been deficient in growth factors, which are normally supplied by yeast autolysate. It is generally believed that bees are responsible for the initial yeast contamination of honey.

Initial multiplication of yeasts in commercial honeys, according to Fabian & Quinet (40), is favored by surface dilution of honey by hygroscopicity after which sugar tolerant yeasts soon adapt themselves to higher sugar concentrations in the deeper layers. Other workers [see Lochhead & Heron (103)] believe that lowering of the concentration of soluble solids by the more readily crystallizable glucose in honey favors the development of yeast, while other factors such as sucrose content (lower osmotic pressure) and nitrogen content are also important. Fabian & Quinet (40) isolated and studied twenty-five yeasts from twenty samples of honey from widely different areas of the United States and Canada. All except one belonged to the genus *Zygosaccharomyces*. The most common organism was *Z. nusbaumeri* Lockhead & Heron (16 isolates), which, however, was incorrectly diagnosed as *Z. priorianus* [see Lochhead & Heron (103)]. Other isolates were *Z. japonicus* (3), *Z. mellis*, a new species (4), and single isolates of *Z. barkeri* and a "black yeast."

Lochhead & Heron (103) made a very thorough study of microbiological spoilage of honey. Four types of yeast were isolated, all belonging to *Zygosaccharomyces*. The cultures represented *Z. barkeri*, *Z. mellis*, *Z. nusbaumeri*, and a new species named *Z. richteri*. This organism should be checked using standard methods of identification, since most tests were conducted in honey media.

In a later paper Lochhead & Farrell (102) showed *Zygosaccharomyces*, *Schisosaccharomyces*, and *Torulopsis* to be present in normal honey, but only *Zygosaccharomyces* species were able to cause honey fermentation. *Z. richteri* was by far the most common yeast present. The same authors (101) showed that ordinary field soil is not to be considered a primary source of infection of honey by sugar tolerant yeasts. Lochhead & McMaster (104) found that there were no outstanding differences in yeast infection of normal honeys, with respect to their floral origin, although a progressively increasing yeast count was found from the Western to the Eastern Canadian provinces, which corresponded with the tendency to ferment during storage. In their first paper Lochhead & Heron (103) also made a study of the organisms present in flower nectar and reviewed the earlier literature on this subject. Not reviewed

were several papers by Grüss (46, 47, 48) dealing with nectar yeasts from high alpine flowers. He also created a somewhat doubtful new genus of nectar yeasts (47) called *Amphiernia* and reported (46) extensive experiments dealing with genetics and physiological properties of the nectar yeasts. In our laboratory typical *Nectaromyces* forms could be isolated repeatedly from flowers of *Teucreum fruticans*, but not from the many other blossoms investigated.

Sacchetti (171) likewise found almost exclusively species of *Zygosaccharomyces* in Italian honeys. Lodder (105) described a new variety of *Zygosaccharomyces* isolated from artificial honey in Holland under the name *Z. major* var. *threntensis* nov. var. Comparable to honey fermentation is that of maple syrup which Fabian & Hall (39) first studied extensively. Causative organisms of spoiled maple syrup from Michigan and Vermont were placed in seven groups: (*a*) *Saccharomyces aceris-sacchari* nov. sp. four cultures); (*b*) *Saccharomyces behrensianus* (five cultures); (*c*) *Zygosaccharomyces mellis* (three cultures); (*d*) *Saccharomyces monacensis* (*S. carlsbergensis* var. *monacensis*) (five cultures); (*e*) *Zygosaccharomyces barkeri* (two cultures); (*f*) *Zygosaccharomyces japonicus* (one culture); (*g*) *Zygosaccharomyces nussbaumeri* (one culture.)

Krumbholz and co-workers in a number of publications (68, 76, 80, 81, 83) made an extensive study of the yeast flora on grapes from a number of European countries and in particular of overripe berries shriveled on the vine ("Trockenbeerenauslesen") and of musts and concentrates made of these grapes. "Osmophilic" yeasts were found on about half the samples of fresh grapes. Such samples were placed in media containing about 50 per cent sugar, and after fermentation was observed pure cultures were obtained by plating.

"Osmophilic" yeasts were particularly prevalent on the "Trockenbeerenauslesen." Identification showed that they belonged principally to the genus *Zygosaccharomyces* of which a number of new species was described. A new few sugar tolerant species of imperfect yeasts were also found which the German workers classified as *Saccharomyces*, but which were renamed *Torulopsis* by Lodder (105) because of the absence of sporulation. She also checked the new species of *Zygosaccharomyces* and the following were accepted as valid: *Z. polymorphys* (including f. *typica*, f. *stellata*, f. *craterica*); *Z. globiformis* (f. *typica*); *Z. variabi-*

lis; Z. gracilis (the latter from a diseased Portuguese white wine). One culture termed *Z. amoeboideus*, by Kroemer and Krumbholz, was renamed *Z. cavarae* var. *amoeboideus* by Lodder. Sacchetti (172) studied the yeast flora of fermenting grape concentrate in Italy and described *Z. gracilis* var. *italicus* nov. var. and *Z. felsineus* nov. sp. Lodder described *Z. citrus* nov. sp., which was originally isolated from fermenting citrus lemonade syrups. Phaff & Douglas (152) found *Z. mellis* in a number of dessert wines and in Haute Sauterne which had developed a turbidity or sediment consisting to a large extent of a nearly pure culture of this yeast. The alcohol content of the dessert wines was about 19 to 21 per cent with a sugar content of 7 to 12 per cent, while the Sauterne contained about 12 per cent of alcohol.

Baker & Mrak (5) found that yeasts were commonly associated with "sugared" dried prunes and figs. In the order of their frequency of occurrence: *Zygosaccharomyces*, *Hansenula*, *Saccharomyces*, *Debaryomyces*, and *Zygopichia* were found. Their work was reported later in somewhat more detail (125). Mrak, Phaff & Vaughn (131) studied the yeasts causing a slow incipient fermentation type of spoilate in dates after drying. They reviewed previous work done on this important spoilage problem and showed that the ratio of *Zygosaccharomyces* to *Saccharomyces* cultures isolated was about 15:1. The *Zygosaccharomyces* were the most sugar tolerant, although *Hanseniaspora melligeri*, which was second in abundance, was able to ferment 50° Brix date syrup. The same was true of *Torulopsis dactylifera*, a new species, which was described. *Hanseniaspora melligeri* was first isolated by Melliger from Egyptian dates and accurately described and named by Lodder (105). It is interesting that the authors have since isolated numerous cultures of this organism in California from dates, fresh figs, a spoiled apple, and other sources but have rarely encountered other species of *Hanseniaspora*. Bedford (9) found *Hansenula subpelliculosa* nov. sp. in a concentrated sugar-egg mixture undergoing spoilage.

Fresh fruits.—Decomposing fresh fruits have been one of the most common sources for isolation of yeasts. Mrak & McClung (128) found a wide variety of yeast genera on grapes and grape products in California, the most common ones being *Saccharomyces*, *Candida*, *Torulopsis*, and *Kloeckera*. The ratio of *Saccharomyces* to *Zygosaccharomyces* was 7:1. Two new species of *Torulopsis* were

described, *T. californicus* and *T. fermentans*. A new species *Asporomyces uval* was also described but later Mrak, Phaff & Smith (130) considered this genus invalid. Ciferri & Verona (29) listed and described all yeasts isolated from grapes, must, or wine in Italy. Mrak *et al.* (132) studied the yeasts found in souring fresh figs and discussed the history of this important spoilage problem. Most of the yeasts were species of *Saccharomyces* and *Candida*, and only one culture of *Zygosaccharomyces* was isolated. A new species *Debaryomyces dekkeri* was described and a variety of *Saccharomyces fragilis* showing striking adaptive lactase formation (in contrast to the type species).

Romwalter & Kiraly (168) propose the theory, on the basis of a number of experiments, that undamaged sound fruit always contains *Saccharomyces* species in the fleshy part, which ultimately causes fermentation during decay. Later Marcus (119) and Niethammer (144) found yeasts in the healthy tissues of a number of fruits and seeds. Marcus found *Torulopsis albida* in gooseberries and *Rhodotorula glutinis* in sour cherries, and Niethammer likewise reported only asporogenous yeasts in various plant tissues. More work seems desirable to verify and expand these studies. Lodder (105) described *Hansenula ciferri* nov. sp. [accepted by Bedford (9)], which according to Ciferri, who isolated the culture, is found very frequently on *Coumarouna punctata*, a tropical fruit of San Domingo. Lodder also described *Saccharomyces pleomorphus* nov. sp. obtained from Ciferri who isolated it from spoiled pineapple. This organism has the interesting property of fermenting sucrose, but not one-third of the raffinose molecule. Castelli (22) isolated and described an interesting new species of *Saccharomyces* from grape must, termed *S. italicus*. It ferments glucose and maltose, but not sucrose, raffinose, and lactose.

Yeasts occurring on meat products.—Mrak & Bonar (126) found that yeasts were in large measure responsible for the occurrence of a slimy or yeasty coating on fresh sausages, when the latter are stored for about a week or less at fairly high humidity. They found that all yeasts isolated belonged to the genus *Debaryomyces* and resembled closely *D. guilliermondii* var. *nova zeelandicus*. Subsequently students enrolled in a yeast course given by the authors have isolated year after year a number of *Debaryomyces* species from a variety of meat products, such as ham, bacon, pickled

meats, various kinds of sausages, and other items. The common occurrence of *Debaryomyces* species on meat products has not been correlated with special metabolic activity of these yeasts.

Yeasts in brines.—Yeasts and yeast-like organisms occur very frequently as films on brines used for the storage of various fruit, vegetable, and meat products. Mrak & Bonar (127) reviewed the earlier literature on brine yeasts. They isolated film yeasts from eight different types of food brines with salt contents ranging from 4 to 20 per cent. *Debaryomyces* species had the greatest salt tolerance and were the most common organisms present. *D. membranaefaciens* and its variety *hollandicus* and *D. guilliermondii* var. *nova zeelandicus* were found and these organisms could be induced to grow in cucumber brine containing 24 per cent salt. Other cultures found were isolates of *Pichia membraneafaciens* and *Mycoderma decolorans*. The latter had a lower salt tolerance and were barely able to grow in 15 per cent salt. Authentic cultures used for comparison showed a somewhat lower salt tolerance, indicating that this property may be reduced when cultures are carried on artificial media for several years. Graham & Hastings (44) isolated and identified yeasts from the pellicle forming on the brine solution used to extract rennet from calves stomachs. They found cultures of *Debaryomyces tyrocola* and *D. guilliermondii*, which appeared to have a somewhat lower salt tolerance, since they grew in 15 per cent salt solutions, but not in 20 per cent salt. The natural source of the contamination of rennet brine is still unknown, since no *Debaryomyces* species could be isolated from the third and fourth stomach of the calf or from various soil samples. We believe that contamination of brine tanks may very well originate from air borne yeasts. Lodder (105) described *Debaryomyces guilliermondii* var. *nova zeelandicus* nov. var. and *D. kloeckeri* var. *major* nov. var., both isolated from rennet in New Zeeland. Hof (63) reported the occurrence of *D. membranaefaciens* and *D. guilliermondii* in salted beans, and Lodder (105) described *D. membranaefaciens* var. *hollandicus* nov. var. isolated from spoiled salted beans. Data dealing exclusively with the tolerance to high concentrations of salts are given in papers by Kroemer & Krumbholz (77), Wheaton (212) and Mrak & Bonar (127).

Yeasts associated with milk products.—Sacchetti (173, 174) studied the yeast flora from Italian soft cheeses and found a high proportion of lactose fermenters, which are relatively of rare oc-

currence on nonlactose containing sugary substrates. Several new species and varieties were described such as *Z. casei* and *Z. versicolor*. Further study and comparison with accepted known species of the sporogenous yeasts appear desirable before accepting these species. Sacchetti considers them to play an important role in the characteristic softening of certain cheeses. Chinn (25) studied the nonlactose fermenting yeasts and yeast-like organisms from cream and butter. The authors have not had an opportunity to read and review this doctorate thesis. Hammer & Cordes (59) studied lactose fermenting yeasts in so-called "yeasty" cream (cream undergoing fermentation during shipment to dairy plants). They described *Torula sphaerica* (accepted but renamed *Torulopsis sphaerica* by Lodder) and *Torula cremoris*, which according to Lodder (106) belongs to the *Mycotoruloideae*. Olson & Hammer (148) studied the yeasts causing gas in sweetened condensed milk. Two *Torula* species described should be rechecked by modern taxonomic methods to evaluate them as new species. Olson & Hammer (149) after reviewing previous taxonomic studies on *Sporobolomyces* and *Bullera* described thirty-two cultures of these genera isolated from the atmosphere in a dairy plant and one isolate from fermented cream. In general *Sporobolomyces* was not common on spoiled dairy products. *Sp. pararoseus* nov. sp. was described and the description of *Bullera alba* was extended.

Yeasts associated with insects.—Grosmann (45), and soon afterwards Rumbold (169), noted the regular association of yeasts and yeast-like organisms with the blue staining fungi isolated from the wood of beetle infested trees. Subsequently Holst (65) showed that a new species *Zygosaccharomyces pini* could be isolated regularly from the bark beetles *Dendroctonus* and *Ips* from various parts of the United States and from the wood infested by them. This yeast is characterized by the formation of hat shaped spores, which are not common in the genus *Zygosaccharomyces*. Holst pointed out the great variability in percentage and rate of sporulation between the different isolates and found that a tendency existed to lose the ability to sporulate. Holst (65) cited a number of similar studies but descriptions of the yeasts were not sufficient for exact identification. Apparently there is no symbiotic relationship between the yeast and the beetles.

Rumbold (170) studied the fungi carried by certain bark beetles and observed the presence of *Z. pini* and several *Candida*

species (termed *Monilia* in her paper) in two species of *Dendroctonus*. Rumbold found that the yeasts promoted the growth of *Ceratostomella* on wood, with which they were associated. *Endomycopsis bisporus* appears to be commonly associated with bark beetles or may be found in the tunnels these insects make. This organism was first found by Beck in *Ips typographus* attacking spruce in Austria (8), and again on the same beetles and trees in Poland about twelve years later by Siemaszko (185). More recently Verrall (203), in Southern United States, isolated the same organism from adults of *Platypus compositus* and from wood adjacent to their tunnels in pecan (Carya species), sweet gum (Liquidambar Styraciflera), Swamp tupelo (Nyssa biflora), and locust (Gladitsia). Verrall apparently was completely unaware of the earlier papers [including Stelling-Dekker (194)] pertaining to *E. bisporus*, for he described it as a new species and termed it "*Endomyces bispora*."

Webb (209), in a thorough paper on the Australian ambrosia fungi, reported that two species of Endomycopsis (termed forms A and B) could be isolated constantly from the tunnels, larvae, and adults of the wood-boring Australian ambrosia beetle *Platypus subgranosus* in Myrtle beech (*Nothofagus cunninghamii*), *Eucalyptus goniocalyx*, and mountain ash (*E. regnans*). Only morphological characters of these two yeasts were given. Steinhaus (193) has reviewed the biologic relationship of yeasts with respect to their host's tissues. It is apparent that the identity of most yeasts from insects is very obscure or doubtful, although the presence of yeasts and yeast-like organisms has been well demonstrated in a large number of insect and tick species. Two yeast genera with needle shaped ascospores, included by Stelling-Dekker in her monograph, have not been cultivated on artificial media and merit restudy. *Monosporella*, an extracellular organism, has been observed to parasitize the body cavity of *Dasyhelea obscura* larvae; and *Coccidiascus*, an intracellular organism, was seen to multiply in the cells of the mid-intestine of the muscid *Drosophila funebris*.

Plant pathogenic yeasts.—Some yeasts are recognized as plant pathogens, in particular species of *Nematospora* and *Ashbya*. There is a large literature describing the occurrence of species of these genera on a number of host plants since they were first isolated from these sources [see Stelling-Dekker (194) for summary]. Most

of the Annual Reports of Agricultural Experiment Stations in tropical and subtropical countries include information on infections of cotton (Internal Boll disease), lima beans (Yeast spot), citrus fruits (inspissosis), coffee berries (Coffee bean disease), and others by various insect vectors. Other sources of information are the Progress reports from Experiment Stations of the Empire Cotton Growing Corporation in London. Leach in 1940 (91) reviewed the role of insects in transmission of plant diseases. Wallace (206) reviewed the subject and has compiled a list of the available records up to 1937 of "Stigmatomycosis" or bug carried diseases, giving host plants, insect vectors, and localities. Points of interest are: (*a*) the widely separated plant families in which susceptible species have been reported; (*b*) the wide geographical distribution of the disease in crops such as cotton and legumes and the restricted distribution in others such as coffee; (*c*) the considerable number of wild host plants (herbs, shrubs, and trees), which serve as reservoirs for bugs as well as fungi. Control measures were also discussed. Muller (133) in a review of the most important citrus diseases in the Dutch East Indies included *Nematospora* rot and pointed out that larvae and insects which have become infected with *Nematospora* once remain contaminated for long periods of time even though no reinfection takes place and the insect feeds only on healthy fruit after the initial infection. Weber (210, 211) reported on the occurrence and pathogenicity of *N. coryli* in Florida and found it on sweet pepper pods, satsuma oranges, grapefruit, oranges, tomatoes, and pecan nuts. *Ashbya gossypii* (syn. *N. gossypii*) is of much less common occurrence and was isolated only once from a satsuma orange, in which it caused the same symptoms as *N. coryli*, a local drying out and collapse of the juice sacs, wrinkling of the rind with slight protrusion of the oil glands above the surface, and a whitish to reddish-brown discoloration of the locule covering. Krug (78) studied the internal boll rot of cotton in the state of Sao Paulo, Brazil, caused by *N. coryli*, *A. gossypii*, some bacteria, and *Penicillium* species and summarized available information on the history, host range, distribution, economic importance, symptoms, and etiology of this disease from six Experiment Stations in that area. *N. coryli* was isolated at Campinas from cow peas from which it may be transmitted to cotton by *Nezara irridula*. Peglion (150) found *Nematospora coryli*

in insect infested maize in Italy. The asci were particularly abundant in the embryo of the kernels and least common in the aleurone layer. The organism was accompanied by other fungi.

Yeasts associated with deterioration of wood and wood pulp.—Wood pulp for paper manufacture is easily attacked by fungi and certain yeasts. Melin & Nannfeldt (122) gave a detailed account of their work on incidence, etiology, and control of blue staining of wood pulp in Sweden since 1929. Besides numerous fungi they found *Pullularia* species commonly in or near blued wood. They suggested reducing infection by seeding grinding water with species of *Mycotoruloides* and *Torulopsis*, which were shown to have inhibitory action on the development of blueing fungi.

Rennerfelt (164) gave an exhaustive and well-tabulated review of his work on the fungal blueing of ground wood pulp in Swedish paper mills. The yeasts or yeast-like organisms regularly associated with the numerous fungi (listed) were *Pullularia pullulans*, several species of *Rhodotorula*, namely, *R. glutinis*, *R. mucilaginosa*, and *R. gracilis* nov. sp. The latter appears to be a valid species on the basis of differences in utilization of sugars and nitrogen sources. Other organisms included *Torulopsis stellata*, *T. aeria*, and a species of *Candida*. The inhibitory effect of certain species of the *Torulopsidaceae* on fungal blueing was confirmed to some extent, although it was pointed out that some species may actually form growth promoting substances. A better way to reduce infection appears to be raising the temperature of the backwater to 45° or 50°C. Goidanich, Ciferri & Redaelli (43) isolated a number of yeasts from stored wood pulp for paper making in Italy, two of which have been known to be associated with human diseases, namely, *Mycotorula* (*Candida*) *zeylanoides*, and *Mycotorula mucinosa nobis* (which may constitute a new species). Other organisms include *Torulopsis albida*, *T. minor*, *Sporobolomyces shibatanum* and *Sp. salmonicolor*. Rennerfelt (165) has also reported the optimum and maximum growth temperatures of a number of fungi and yeasts isolated from wood pulp.

Yeasts from mushrooms.—Anderson & Skinner (2) found and identified a number of yeasts in autolyzing mushrooms and some fleshy *Ascomycetes*. They feel that the organisms are carried to mushrooms by insects. Yeasts are especially prevalent in fungi yielding an acidic autolysate. Forty-five cultures of *Saccharomyces*

cerevisiae (various varieties): four *S. chodati;* twenty, *S. dairensis;* two, *S. disporus;* four *S. heterogenicus;* nine, *S. muciparis;* and six, unidentified *Saccharomyces* species were isolated from numerous fungi. Other organisms found were: twenty-two, *Torulopsis pulcherrima;* one, *T. utilis;* five, *T. uvae;* one *T. californicus;* one, *T. sphaericus;* one, *Rhodotorula glutinis;* and six, *R. colostri.* It is noteworthy that the one hundred and seventy isolates represented only three genera of yeasts. Forty-five cultures were presumed to be smut sporidia, only outwardly resembling yeasts.

Yeasts occurring in soil.—Although the soil presumably constitutes an important reservoir in which the yeasts may survive during the winter or other periods of unfavorable conditions, very few reliable taxonomic and ecological studies have been made. Starkey & Henrici (192) reviewed the literature up to 1927. They found yeasts in thirty-nine of eighty-seven soil samples, taken during various seasons and from various types of land, but the yeasts were present in such small numbers that they were believed not to be involved in soil transformation. Twelve different species were isolated, of which only three were positively identified, namely, *Nadsonia fulvesens, H. anomala,* and *R. glutinis.* In addition an organism was found showing bud fission, but no sporulation. A separate genus was later created for this organism by Ciferri (27), who named it *Schizoblastosporion* (species *Starkeyi-Henricii*). Other genera found were *Zygosaccharomyces, Torulaspora, Saccharomyces, Pichia,* and *Torulopsis.*

Ciferri (26) also found yeasts in soil, from the Dominican Republic, in very small numbers as compared to bacteria and other fungi. He found that a large percentage of vegetative yeast cells planted in soil died off within a week. Nissen (145) investigated ten physically and chemically different soils, taken during four seasons. He found that about 50 per cent of one hundred and sixty samples contained yeasts consisting of species of *Saccharomyces, Hansenula, Candida, Torulopsis, Rhodotorula,* and one apiculate yeast. He found that the type of soil had a strong influence on the occurrence of yeasts, fruit orchard soil having the highest and fir forest soil the lowest numbers. The season of the year also had a marked effect on the number of yeasts present. Lochhead & Farrell (101) studied soil as a source of infection of honey. They found only sugar tolerant yeasts in apiary soils, but

not in ordinary field soil. They isolated and described one hundred and sixty-six cultures. Six species and one subspecies were identified.

Bouthilet (16) using modern methods of classification made a thorough study of eighty-four Minnesota soil samples (fruit orchards excluded) collected in the spring and found that all samples yielded yeasts; but the survival of culture-grown yeasts inoculated in soil was very poor. All his samples yielded yeast which appeared to be principally oxidative in character. Eighty per cent of the one hundred and twenty isolates could be placed in *Hansenula*, *Candida*, and *Rhodotorula* while the rest consisted of species of *Debaryomyces*, *Torulaspora*, *Pichia*, *Mycoderma*, *Schizoblastosporion*, *Torulopsis*, and *Trichosporon*. *Zygohansenula californica*, originally isolated from California soil and first described by Lodder, was again isolated from soil by Bouthilet in his survey.

Yeasts in ocean water.—Zobell (234) reviewed the literature available on the occurrence of yeasts in the sea and in lakes. Unfortunately most of the reports fail to give proper descriptions of the yeasts found, so that identification is hardly possible. In some instances sporulating yeasts of the genera *Endomyces* and *Saccharomyces* were reported, but mostly reference is made to pink and white *Torulae*, *Mycoderma*, *Dematium*, *Oidium*, and a few others. Yeasts seem to be generally present regardless of the distance from land and some samples of the open ocean contained more yeasts than bacteria. A survey of marine yeasts, using modern taxonomic methods, might yield interesting ecological data.

Special fermentations involving yeasts.—Allen & Allen (1) reported on the fermentation of ground taro root for the manufacture of poi, a common food of native Hawaiians. They found that lactic acid bacteria were the principal causative organisms for the main fermentation, which consists of souring, but that yeasts always occur during the final stages. Little or no alcohol is formed. The yeasts were not well identified and were referred to as "*Mycoderms*" and *Oidiums*. A number of unusual fermented beverages, in some of which bacteria and yeasts act together to produce the required end product, have been made for centuries in various countries. One of these, the "Tibi" fermentation, has been studied

most accurately. This popular Swiss drink is a sour, weakly alcoholic carbonated liquid made by the fermentation of a 15 per cent cane sugar solution to which dried figs, raisins, and a little lemon juice have been added. The inoculation is done by adding a number of Tibi grains which consist of a capsulated bacterium and a yeast. The historical facts have been reviewed by Mayer (121), who made a thorough study of the symbionts. The bacterium was identified as *Betabacterium vermiforme* and the yeast as *Saccharomyces intermedius* Hansen. The combined action produces lactic acid, alcohol, and carbon dioxide. The Tibigrains multiply during fermentation and can be transferred to a subsequent batch. Mayer was able to prepare synthetic Tibi grains from pure cultures of the two symbionts. According to this author it appears very likely that the so-called "ginger beer plant" used in England for similar purposes and described by Ward (208) is identical with the "Tibi Konsortium." Koolhaas & Boedÿn (74) reported on a "tea fungus" used in Indonesia to prepare an aromatic, slightly acid drink from tea infusion plus 10 per cent sugar. Two symbionts make up the "fungus"; they are *Bacterium xylinum* (producing the acid) and *Saccharomycodes ludwigii*, a yeast which produces small amounts of carbon dioxide and alcohol. Other bacteria and yeasts appear to be associated with these two organisms. Klöcker (69) mentions a number of other fermented products where symbiosis between lactic acid bacteria and yeasts is involved. For the most part the organisms involved have been studied only superficially and at a time when satisfactory methods of identification were not available. A thorough reinvestigation of these fermentations would be well worth while. Examples are Kefir fermentation (Caucasus), caused by inoculating Kefir grains into milk; Kumis (Asia); Leven (Egypt); and Mazun (Armenia).

A fermentation in which two yeast floras succeed each other occurs in the fermentation of Belgian ale (lambic), English or Irish Ale, and porter or Stout beer. Custers (32) has reviewed completely the literature and studied the causative organisms. The main fermentation is carried out by a typical beer yeast of the *Saccharomyces carlsbergensis* type, while the secondary fermentation, which may take from six months to two years is caused by a number of *Brettanomyces* species. The latter ferment and grow

very slowly and have the ability to convert part of the alcohol into acetic acid when conditions are aerobic. They are primarily responsible for the typical flavor of true ale.

Another group of organisms of interest to the wine industry are the so-called Jerez yeasts or Sherry film yeasts. Hohl & Cruess (64) showed that some of these yeasts are morphologically and physiologically similar to *S. cerevisiae* var. *ellipsoideus*, except that they form a distinct film after fermentation is complete. The film stage causes development of the characteristic bouquet in Sherry. Other biochemical characteristics of these film yeasts were studied. Another group of film yeasts was described by Scrivani (184), although these were isolated as spoilage organisms from Italian wines. Fifty-four samples yielded one hundred nineteen isolates including cultures of *Pichia* and *Zygopichia* and *Mycoderma*. Eight strains of *Mycoderma* could not be identified with existing cultures. A *Pichia membranaefaciens* var. *acidificans* nov. var. was described, although we question its taxonomic position in view of the fact that it is reported to utilize potassium nitrate but not ammonium sulfate. If this observation is correct such a culture should be placed in *Hansenula*, although *Hansenula* species commonly use ammonium sulfate also as a nitrogen source. The various strains differed a great deal in their ability to utilize alcohol and to produce acid. Zimmermann (232) described methods for selection of desirable wine yeasts from a commercial mixed sediment. He isolated and described wine yeasts with special characteristics, such as cold tolerance, high sulfur dioxide tolerance, various rates of fermentation, final alcohol and volatile acid content, and sedimentation properties.

Hall, James & Nelson (58) isolated the causative organisms of the curing process of Barbados "Molasses." At the curing stage this syrup is about 42° Baume. Two types of yeast were found. The first was *Z. nussbaumeri* Lochhead & Heron (five cultures). The second type of yeast isolated consisted of six cultures of *Z. major* Takahashi and Yukawa. (After fermentation, *Cl. saccharolyticum* is thought to be responsible for the production of the typical rum flavor.)

Incidental isolations.—A number of interesting organisms have been isolated from single samples of a variety of spoiled foods or other sources. Wickerham & Duprat (214) isolated a new species termed *Schizosaccharomyces versatilis* from home canned grape

juice undergoing fermentation. A very complete description of this organism was given (See Taxonomy section).

Nickerson (135) isolated *Zygosaccharomyces acidifaciens* nov. sp. from a domestic red wine which had turned sour (pH 2.2). No bacterial spoilage was found and the yeast was apparently present in pure culture and responsible for the souring. It was found to produce acid from alcohol and glucose, and the amount of acid formed increased with increasing glucose concentration (at 30 per cent glucose 0.1 *N* acid was formed). The organism forms the acid principally under anaerobic conditions (in contrast to *Brettanomyces*) and appears to carry out a fermentation similar to the 3[d] Neuberg scheme, forming, besides alcohol and carbon dioxide, glycerol and acetic acid in appreciable quantities [Nickerson & Carroll (140)].

Schnegg & Weigand (181) examined seventeen samples of 3 per cent boric acid solutions obtained from druggists throughout Germany. All were contaminated by microorganisms (444 to 56,800 per ml.), chiefly cultures of *Rhodotorula* and *Pullularia*, while a few *Torulopsis* and molds were also found. The viability of these contaminants was great; one culture of *Rhodotorula* was detected in a three-year-old solution, while other organisms tested did not survive longer than two weeks in such solutions. Sources of contamination are believed to be commercial storage vats. A few years ago the authors also found a bottle of commercial boric acid in California, which contained a pink sediment consisting of a nearly pure culture of *Rhodotorula*.

Candida albicans, although mostly associated with human disease, has been isolated occasionally from nonanimal sources. Redaelli *et al.* (158) found it as a contaminant in samples of home made Italian bakers' yeast. Verona & Ciferri (200) reported it associated with plant pathogenic organisms causing rot in carrots. However, their description does not at all fit the accepted definition of *C. albicans* and it appears that they were dealing with another species of *Candida*.

Lodder (105) described two new *Pichia* species and one new variety. *Pichia fermentans* nov. sp. was first found in buttermilk but has been subsequently isolated by the authors from California fruits. *Pichia neerlandica* nov. sp. was isolated repeatedly from spoiled beer and bakers' yeast in Holland. *P. alcoholophila* var. *naganishi* nov. var. was obtained from Naganishi (substrate un-

known). Although a number of interesting and rather uncommon yeasts, such as *Nadsonia*, *Saccharomycodes*, *Endomyces*, and others, have been isolated in the past from slime flux and other exudates of trees [see Stelling-Dekker (194)], no recent work on the ecology of these organisms has appeared in the literature as far as the authors are aware.

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GENETICS OF THE FUNGI

By Carl C. Lindegren

Department of Biological Science and Research, Southern Illinois University Carbondale, Illinois

Mucors

Genetics of the fungi takes its origin from the discovery by Blakeslee (1) that copulation between two thalli of opposite mating types precedes the formation of zygospores in *Rhizopus*. It is preferable to call this a mating type difference, instead of a sexual difference, for later work on the Basidiomycetes revealed that two pairs instead of a single pair of genes control mating type specificity, which leads to the obvious contradiction that there are four sexes in this class. Furthermore, work with *Neurospora* showed that mating type specificity is superimposed upon true sex, true sex being defined as the existence of male and female sex organs. In view of these complications, the term "sex" should be reserved for sex organs and the term "mating type" for genetic differences controlling cross-copulation or self-sterility.

Blakeslee & Cartledge (54) found intermediate mating type reactions. Satina & Blakeslee (55) showed that one mating type was somewhat richer in reducing substances than the other. Burgeff (2) collected over twenty variants of *Phycomyces Blakesleeanus* and analyzed the results of hybridizing these mutants. The Phycomycetes are called the siphonaceous fungi because of the absence of crosswalls in the mycelium. The gametangia are multinucleate and when the zygospore is cut off, large numbers of nuclei are enclosed in the young zygospore. Copulations are multiple, and many pairs of nuclei fuse in the zygospore, while many haploid nuclei are left over; the latter apparently disintegrate.

Tetrad analysis is not feasible because several diploid nuclei are produced within a single zygospore, and no cells comparable to ascospores, which isolate individual haploid nuclei, are found in Phycomycetes. After hybrid zygospores were formed, Burgeff allowed them to germinate, and the individual haploid spores which were produced in the aerial sporangium were collected and diagnosed. Burgeff made and analyzed numerous crosses. From a doubly heterozygous hybrid, one expects at least four types of haploids from each zygospore. However, this did not occur fre-

quently; many zygospores produced only one, two, or three of the expected types. He did show, however, for the first time that

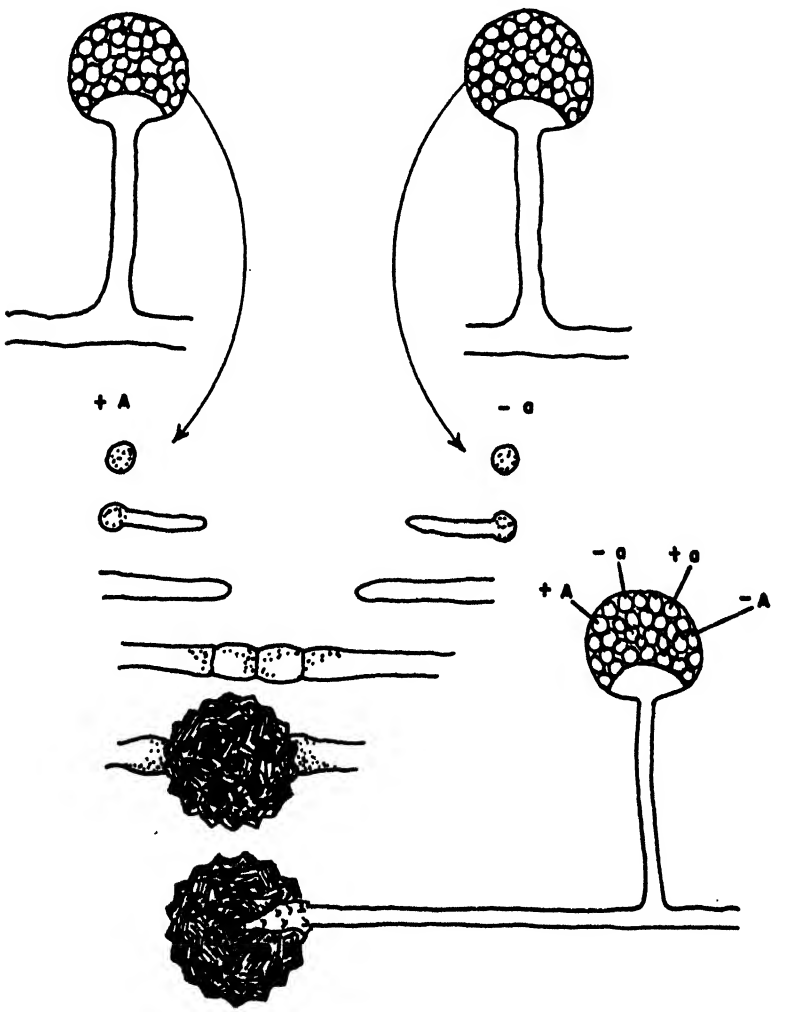

FIG. 1.—COPULATION AND SEGREGATION IN A MUCOR.

segregation in Mucor occurred in both the first and the second reduction division; but he was unable to detect or calculate linkage. Burgeff also discovered the interesting fact that zygospores can be

put away in absolute alcohol and preserved in a viable state for a considerable length of time. His 1927 paper is the most ambitious attempt to analyze the genetics of the Phycomycetes.

SCHIZOMYCETES

Lederberg (3), working with Tatum, discovered that it is possible to mate genetically different mutants of *Escherichia coli* by mixing the cultures together in nutrient medium and plating out on specially designed agars. He discovered fifteen genes in one linkage group, apparently in a linear order, suggesting that all the loci lie on one chromosome. This discovery gives genetical analysis in the bacteria almost the same validity that it has in higher organisms. Prior to this, most of the genetical work in the bacteria was by plating and selecting variants which resemble mutants known to occur in other organisms. However, there is no way of distinguishing these variants from the depletion mutations, recently described in yeast [Lindegren & Lindegren (4)]. A red yeast produced stable white "mutants," but whenever these white "mutants" were outcrossed, they were transformed to red. The hybrid (white "mutant" × normal) produced half red progeny just like the original red which, when mated to normal, also produced half red progeny. Therefore, "mutation" from red to white was completely reversed in the heterozygote. This experiment raises the question of how many of the mutations in bacteria are of this type. Lederberg's work suggests that regular segregations of some bacterial mutations can occur.

Witkin (5) has found that many hitherto unsuspected chemicals are capable of producing mutation, even common table salt being a mutafacient substance. Wyss, Stone & Clark (6) have shown that it is possible to induce mutations and with a much higher frequency by pretreatment of broth with hydrogen peroxide or ultraviolet light. This discovery suggests that the induction of mutants resistant to antibiotics may be capable of precise control, but has revealed no suggestions concerning the mechanism by which induction occurs. Miller & Bohnhoff (7) found that when the meningococcus is exposed to streptomycin the percentage of resistant mutants is proportional to the concentration of streptomycin. If the frequency of mutation is a function of the concentration of an inducing substance, new methods of attacking the problem of the induction of mutations may be possible.

Hymenomycetes

The Hymenomycetes include the mushrooms and toadstools. They grow from an underground mycelium to produce a mushroom fruiting body on whose gills (hymenia) the basidia are borne. Generally, up to the time of production of the basidium, the cells are dicaryons with two separate complementary haploid nuclei in each cell. In the basidium, the nuclei fuse, undergo reduction, and produce four basidiospores on each basidium. The four spores from a single basidium can be collected by making a "spore print" and tetrad analysis of the fungi had its origin in the development of this technique by Kniep (8). Kniep discovered that many toadstools have tetrapolar "sex" and showed that the mating type alleles are Mendelian characters.

In the classical case, the zygote is heterozygous for two pairs of genes and has the constitution Aa Bb. There are three types of basidia: (*a*) AB, ab, Ab, aB; or (*b*) AB, AB, ab, ab; or (*c*) Ab, Ab, aB, aB. Each basidiospore produces a mycelium which can be mated with a complementary mycelium to produce a dicaryotic (often incorrectly called a diploid) mycelium according to the scheme below.

	AB	ab	Ab	aB
AB	—	Clamps	—	Barrage
ab	Clamps	—	Barrage	—
Ab	—	Barrage	—	Clamps
aB	Barrage	—	Clamps	—

Bensaude (9) discovered that the clamps (which indicate a dicaryotic mycelium) insure the production of cells, each of which contains two complementary nuclei.

Vandendries (10) discovered that when two mycelia with common B factors are in opposition, they grow to within a few millimeters of each other and then stop without mingling due to some inhibiting substance. He named this phenomenon "barrage."

Brunswik (11) made an elaborate study of mating type specificity in about twenty species of Coprinus. He found great differ-

ences from the classical type of clamp connections, some species being without clamps until the formation of the basidium, while others have precise and regular clamp connections throughout the life cycle. He discovered many irregularities in the behavior of mating types, and this work was much extended later by Vanden-

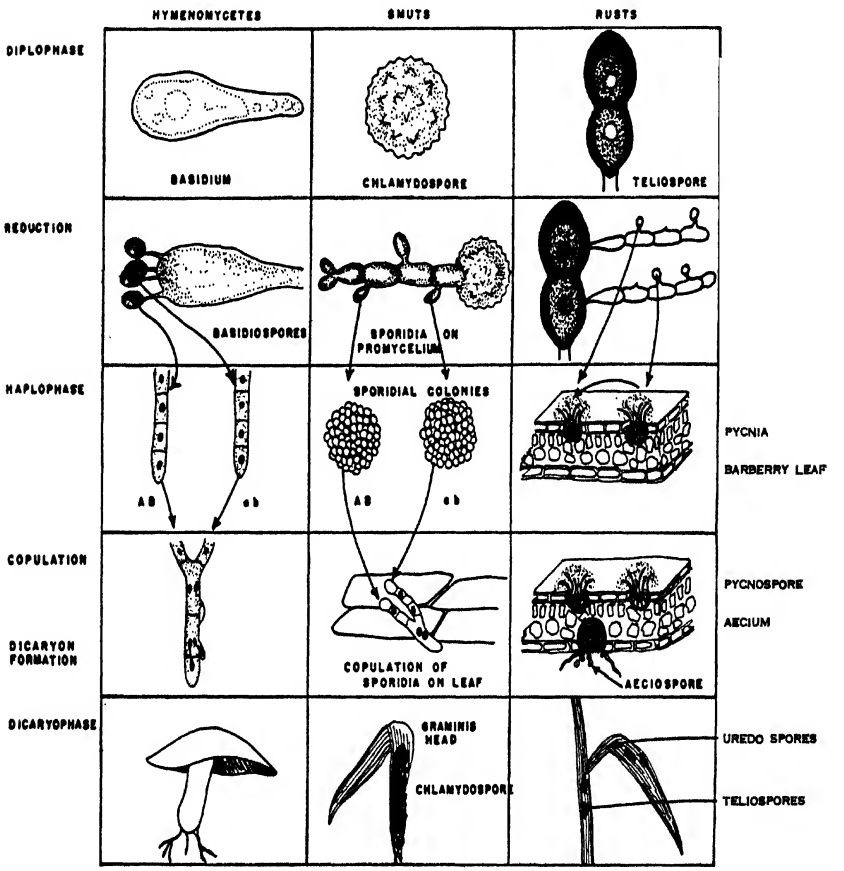

FIG. 2.—LIFE CYCLES IN THE BASIDIOMYCETES.

dries (10) and Hanna (12). Two mycelia of the same mating type often copulate and produce clamps, due to an "illegitimate" mating ("Durchbrechungskopulation"). Cultures grown from spore prints from a single fruiting body may interact with great regularity according to the tetrapolar scheme, but when a culture from

a toadstool collected a few yards away (whose spores likewise behave regularly *inter se*) are tested, the two groups from the neighboring dungheaps are often found to be completely interfertile. This has been interpreted as due to multiple allelism of the genes A and B. Collections of mycelia originating from different continents when cross-mated often reveal much sterility.

Buller (13) discovered that a fully grown haploid AB mycelium in contact with a haploid ab mycelium is invaded by ab nuclei and becomes a dicaryotic mycelium. Migration of ab nuclei through the AB mycelium apparently accounts for this effect. The AB mycelium can also be dicaryotized by an Ab/aB dicaryon, although neither an Ab nor an aB mycelium can achieve this effect. Since only an ab haplophase can dicaryotize the AB mycelium, this phenomenon is puzzling. Many proposals have been made concerning the mechanism by which the reaction occurs, but none is completely satisfactory. My own preference is for an explanation which I have not seen published, but which Quintanhila (51) proposed in the course of a conversation: either the aB or the Ab nucleus in the dicaryon is converted into an ab nucleus by a directed mutation similar to that which occurs in the transformation of pneumococcal types.

Harder (14) produced a dicaryotic mycelium by mating two variants of *Schizophyllum commune* and operated on the dicaryon to produce a haploid mycelium which contained the nucleus of one variant in the mixed cytoplasm of the two variants. This is easily achieved by cutting off the clamp of the penultimate cell before it fuses with the ultimate cell and isolating the mononucleate ultimate cell by cutting through the penultimate cell. He discovered that the addition of cytoplasm produced a stable change in the culture. The operation produces an X nucleus in X+Y cytoplasm, or a Y nucleus in X+Y cytoplasm. These cultures can be compared with the original haploids: X nuclei in X cytoplasm and Y nuclei in Y cytoplasm. The additional cytoplasm produced stable mutants. Harder interpreted this as a cytoplasmic effect. In view of the fact that McCarty, Taylor & Avery (15) and Boivin, Delaunay, Vendrely & Lehoult (16) have shown that simple chemical substances can produce stable transformations, Harder's conclusions may require qualification. The experimental procedure which Harder used to produce his "operated" variants is quite simple, and it would seem that this is one of the important discoveries in the Hymenomycetes that could be profitably repeated.

Smuts

The smuts are parasites on grassy plants and are economically important because they attack corn and small grains. The seeds of an infected plant are replaced by a sooty dust called smut; the soot is composed of chlamydospores, each of which contains a diploid fusion nucleus. In the spring a reduction occurs and the chlamydospore germinates to produce a promycelium containing four nuclei. The promycelium is divided into four cells and sporidia bud from each of the cells. Sporidial cultures resemble yeasts and can be grown in artificial media. There are two mating types in most smuts and sporidial cultures copulate to produce the dicaryons which infect the growing plant, which is almost always parasitized by a dicaryon. *Ustilago levis* attacks only the oat plant while *U. hordei* attacks only barley. The dicaryon produced by the copulation of *U. levis* × *U. hordei* attacks both. Among the tetrapolar smuts, Bauch (17) discovered that an AB/ab dicaryon on an agar plate produces "Suchfaden" which are recognized by their rapid filamentous extension across the plate; they correspond to the dicaryotic hyphae which infect the host. When two sporidial cultures with common A factors but different B factors are mated, "Wirrfaden" grow out of the mixed culture on the agar plate. "Wirrfaden" are twisted copulation tubes resembling weak, distorted "Suchfaden." Bauch discovered that both the A and B mating type factors exist in a multiple allelic series producing a great variety of pathogens, and that the same alleles were present in cultures isolated from England, Germany, and Siberia.

Bauch found that the chlamydospores from each naturally infected plant were all of one genotype, but that neighboring plants contained genetically different fungi. Christensen (18) made an extended study of the problem of solo-pathogens. In yeast they would be called illegitimate diploids; solo-pathogens are monosporidial cultures which are capable of infecting the plant by producing a diploid or dicaryotic mycelium which grows throughout the plant and finally produces chlamydospores. The chlamydospores do not, however, always produce solo-pathogenic sporidia.

Chlamydospores are rather easy to collect and can be germinated on agar. Hüttig (19) has classified the types of promycelia produced by the different types of smuts. There are several variations from the classical type. In one, the promycelial cells send out germ tubes instead of producing sporidia. These germ tubes copulate with each other and, by this means, it is possible to dis-

tinguish first- and second-division segregation by direct inspection. These varieties have bipolar mating type. If we number the cells of the promycelium 1, 2, 3, and 4 from the tip downward, there are two types of copulations: (*a*) the germ tube originating from 1 may copulate with 2, and the one originating from 3 may copulate with 4, or (*b*) the germ tube originating from 2 may copulate with 3 and that originating from 1 may copulate with 4. These are the only types of copulations which are found. On the basis of calculations derived from observations of this phenomenon, Hüttig (19) found that the normal ratio of first- and second-division segregation was 23:77.

In *Neurospora* Lindegren showed that the maximum expected frequency of second division segregation is 67 per cent, and this figure is not exceeded in the analysis of the segregation of a large number of factors. This depends on the assumption that the chromosomes cross over freely along their entire lengths. The high frequency of post reduction in *Ustilago* found by Hüttig is of considerable theoretical interest. His calculations are based on the assumption that copulation is between germ tubes originating from cells of opposite mating type and although confirmation of this assumption is not presented, it is difficult to criticize the point of view. Hüttig plated the chlamydospores on a medium containing a large variety of chemicals and found in every case that second-division segregation occurred in excess of expectation. A possible explanation of the apparent discrepancy with *Neurospora* was suggested in a conversation with Dr. McClintock: the chromosomes in *Ustilago* may form a pair of chiasmata spanning the centromere, with only a single cross over occurring in each arm of each chromosome (52). This would make possible a 100 per cent post reduction depending upon the position of the locus.

Several attempts have been made to study the segregation of colonial characters such as color and colony shape in the promycelia of smuts. To study the segregation in *Ustilago*, it is necessary to inoculate the living host with paired cultures and collect the chlamydospores resulting from the infection. None of the workers has attempted to interpret the results in conventional genetical terms, although much of the data is obviously susceptible to this approach. The best data are those of Dickinson (20), who studied the segregation of five characters. He only analyzed a limited number of promycelia, but my calculations of his data indicate that all five factors are in a single linkage group, although the data are not

statistically significant. This suggests that the smuts may be excellent genetical material, in spite of the fact that they have not been exploited to this end, presumably because the workers in this field are more directly concerned with problems of economic importance. It may be, however, that these problems could be more quickly solved if a genetical analysis of the organism were available. The photographs presented by Stakman *et al.* (21) show that this analysis would not be difficult.

An idea which has persisted in smut literature is that segregation may occur in the third division. This idea is based primarily on the extreme variability of many of the sporidial cultures. I believe that if one analyzed the data on the assumption that this variability was due to mutation and that third-division reduction does not occur, a linkage map could readily be developed, in spite of the fact that progress to date has been disappointingly slow.

The cytological evidence presented in support of the third-division segregation is quite irrelevant. Four chromosomes have been reported on the metaphase plate and an occasional third division has been observed in which two chromosomes were reported going to the poles. Since the haploid number of chromosomes always appears at the metaphase plate of meioses I and II (due to synapsis), there are at least four chromosomes in the haplophase and the supposed observation of two chromosomes at a later division can only be due to inability to resolve small closely approximated bodies. That this must occur frequently is shown by the failure to find four chromosomes always going to the poles in meioses I and II.

The fact that parasitism on the host only occurs when two sporidia of opposite or complementary mating type copulate and produce a dicaryon suggests that complementary genes linked to the mating type genes are essential for production of infective hyphae. The complementary genes must exchange infrequently, a condition which could be achieved by the presence of an inverted section (in which crossing over was inhibited) adjacent to the mating type alleles. Complementary pathogenicity genes would be expected to accumulate in such an inverted section.

Rusts

The rust fungi comprise thirty-five hundred known species attacking a great variety of plants. At least two hundred distinct strains of wheat rust are now known, and it seems probable that

an equal variety may exist for each of the thirty-five hundred species. Because of the great economic importance of wheat rust, there has been abundant support for research on the genetics of rusts. Wheat rust is a characteristic heteroecious rust spending part of its life cycle as a parasite on the barberry and part as a parasite on wheat. The sexual cycle takes place on the barberry while the economically important wheat rust represents the vegetative phase of the hybrids produced on the barberry. The barberry is the reservoir of variability. Control of the disease by the development of resistant wheat varieties has not been successful to date because the new hybrids arising from the barberry have always been effective against any previously resistant wheat. There are two difficulties in the analysis of heredity in rusts: (*a*) these fungi are obligate parasites and have not as yet been grown on artificial medium, and (*b*) the only forms which can be characterized are the dicaryotic hybrids growing on wheat, i.e., haplophase cultures cannot be studied, thus one of the principal advantages of fungal genetics is not available in the rusts.

There were four important steps in the history of rust genetics:

(*a*) DeBary showed that the pustules on barberry and the rust on wheat are both caused by the same organism. He even recognized the true significance of the pycnospores as sperm and although his ideas were disregarded for fifty years, they were finally abundantly confirmed by Craigie (22). DeBary's genius is one of the highlights of the nineteenth century.

Eriksson (56) was the first to describe physiological races of rusts. Stakman & Piemeisel (23) developed techniques for characterizing the different strains parasitic on wheat by infecting different varieties of wheat with a given strain of rust and distinguishing the different strains of rust on the basis of their effect on different wheat varieties. A rust strain may be extremely virulent with regard to one wheat variety and relatively ineffective on the second. There are no rusts that are virulent against all strains of wheat, and there are no wheat varieties that are resistant to all types of rusts. Stakman and his co-workers have catalogued numerous biological races by testing different wheat varieties and are principally responsible for the present census of two hundred biological races of wheat rust.

(*c*) Craigie (22) discovered that rusts are heterothallic. He found that the small pustules produced on the upper surface of the barberry leaf by the infection of the sporidia actually function as

sperm, and that the so-called pycnospores can be used to fertilize strains of opposite mating type. The pycnospores are homologous to the spermatia of *Neurospora;* each sporidial culture on the barberry is itself self-sterile but can be cross-fertilized by transfer of pycnospores from a pycnium produced by a sporidium of complementary mating type. In nature, cross-fertilization is achieved by insects.

(*d*) Newton & Johnson (24) perfected techniques for the growth of the rust on barberry and artificial hybridization under controlled conditions. They demonstrated that pathogenic characters are segregated in a Mendelian manner, and Johnson & Newton (24) discovered evidence of maternal inheritance of some characters. Occasional reciprocal crosses produced different kinds of hybrids, depending upon which parent contributed the sperm, indicating that some characteristics cannot be transmitted through sperm.

At present, the rusts are not promising material for the solution of fundamental problems in genetics, because the only characters available are the teliospore color and pathogenicity of the dicaryotic hybrids and tetrad analyses cannot be made. Therefore, they are inferior to smuts which produce similar promycelia, but whose sporidial cultures can be grown on artificial medium. The most important problem in rust genetics is obviously the development of an artificial medium for the growth of sporidial cultures, and considering the present state of our knowledge of culture media, this should not be a difficult task. Its solution would immediately open up the field for tetrad analysis.

Stakman and his co-workers have found that the principal source of new hybrids is Mexico and the Southwest, where virulent pathogens develop through the sexual cycle on the barberry. From here they are disseminated in the summer by vegetative reproduction and the prevailing winds to the Northern United States and Canada.

Saccharomyces

The cells of *Saccharomyces cerevisiae* exist in both haplophase and diplophase. There are two mating types in the haplophase, and the haploid cells of different mating type copulate to produce diploid cells. Kruis & Satava (25) in Prague and Winge (26) in Copenhagen showed that the standard vegetative cells are diploid, produced by copulation of two spores or gametes derived from

spores. The diploid nuclei undergo reduction at spore formation to produce four haploid ascospores. The large, ellipsoidal vegetative yeast cell is produced by the fusion of two round haploid gametes derived from ascospores. Winge established the basic facts of this life cycle by a classical series of observations on the germination of ascospores and fusion of haploid cells. Winge & Laustsen (27), in a series of notable papers, showed that colonial characteristics, fermentative ability, and cell shape are under the control of genes which segregate at the reduction division. They hybridized yeasts by placing a haploid ascospore from one strain in close proximity to an ascospore of a second strain by use of the micromanipulator. When all conditions are favorable, the two spores fuse to produce a diploid hybrid cell.

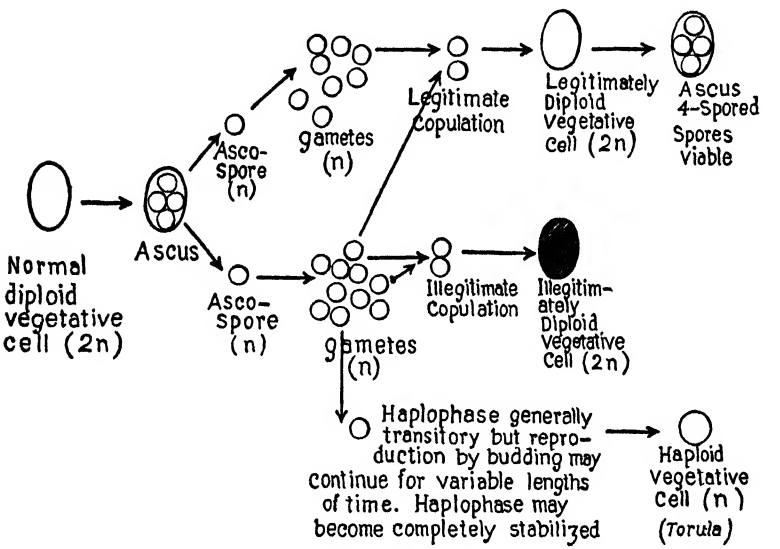

FIG. 3

Lindegren & Lindegren (28) developed a method for hybridizing yeasts based on the fact that single ascospores of *S. cerevisiae* usually produce persistently haploid cultures. It is possible to hybridize these with other similarly derived haploid cultures by mixing the cells together in an appropriate medium. The mixtures result in copulation if each culture is paired with a complementary type. One parent culture can be mated to a large number of other clones. The parents can be classified for biochemical and other

characteristics previous to the matings and the haploid progeny can be classified subsequently. Lindegren & Lindegren (50) have developed stocks heterozygous for genes controlling the fermentation of galactose, sucrose, maltose, melibiose, and raffinose. They have also developed stocks differing in their abilities to synthesize the B vitamins biotin, thiamine, *p*-aminobenzoic acid, pantothenate, pyridoxine, nicotinic acid, and inositol. These cultures are all interfertile and over two thousand hybrid asci have been analyzed. Several cases of linkage have been discovered and maps have been constructed.

Lindegren & Lindegren (29) discovered that haplophase cultures of *S. cerevisiae* fall into two mating types which they have designated a and α. Each haploid culture is made up of potential gametes which can be mated with other cells of appropriate genetic composition from another culture. As the pure haplophase cultures age, illegitimate copulations occur in some cultures between two cells of the same reaction in the same culture, but these matings rarely result in the production of characteristically large diploid cells. Diploid cells produced by illegitimate copulations are generally distinguished by smaller size and diminished ability to produce four-spored asci containing viable spores. Legitimate matings between a and α haplophase cultures derived from a variety of strains of *S. cerevisiae* usually produce large diploid cells which sporulate to form four-spored asci containing viable spores. The procedure is as follows: 1 cc. of broth is placed in a six by three-fourths inch tube. The broth is first inoculated with a large loop of cells from an agar slant of the tester culture and shortly thereafter with a large loop from the haploid culture to be tested. Copulations sometimes appear within six hours. The tube is kept overnight in an incubator at 19°C., and inspected under the microscope for the presence or absence of copulations.

The technique of mass mating has been criticized by Winge & Roberts (63), but Lindegren & Lindegren have established the validity of their technique by analyzing hybrids heterozygous for as many as nine pairs of alleles and showing that all the tetrads had arisen from hybrids and none from illegitimate or selfed diploids. Moreover, even if illegitimates are produced, they are immediately detected when both parents are well marked genitically.

The fact that the haplophase culture from a single spore can be used for an indefinitely large number of matings is particularly important when one wishes to introduce new genes into a stock.

Only occasional ascospores isolated from natural species of *Saccharomyces* produce gametes which copulate with members of our inbred compatible stocks. These rare clones are used to bring new characters into the pedigree.

Some haplophase mutants obtained by continued selection involving numerous platings and transfers over a prolonged period (usually more than a year) were found to be incapable of copulation. Prolonged competition and selection probably result in loss of fertility because the genes which insure fertility do not have a high survival value in competition with other rapidly growing mutants.

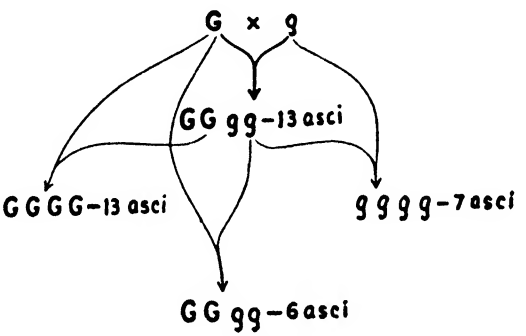

FIG. 4

Figure 4 summarizes data on the inheritance of galactose-fermenting ability in one pedigree. No exceptions to standard Mendelian inheritance were found. Thirteen asci were analyzed from a heterozygous hybrid made by mating a galactose fermenter (G) by a nonfermenter (g); two spores in each of these asci carried the dominant gene controlling fermentation of galactose, and two carried the recessive allele. A backcross of fermenter to the fermenter parent produced thirteen asci; all four spores in each of these asci carried the fermenting gene. A backcross of the nonfermenter to the nonfermenting parent produced seven asci, each of which contained four nonfermenting spores. A heterozygous zygote was produced by back-crossing a nonfermenter to the fermenting parent; six asci were analyzed and each contained two fermenting and two nonfermenting spores. This analysis shows quite convincingly that in some pedigrees the genes controlling fermentation of galactose may behave in a regular Mendelian manner. In contrast to the regular behavior of the genes controlling

galactose fermentation, irregular behavior occurred when *S. cerevisiae* haploid (nonfermenter of melibiose, m) was mated to a *S. carlsbergensis* haploid (melibiose fermenter, M). Several other examples of irregular, non-Mendelian segregations were described by Lindegren & Lindegren (29) which have been explained (after the elimination of other possibilities) by the transfer of material from one chromosome to the other producing stable transformations comparable to those known to occur in the pneumococcus and the colon bacillus. They conclude that the chromosome carries a synthesizing mechanism which can be transferred from an active to an inactive locus.

A haplophase of *S. cerevisiae* was subjected to mustard gas treatment by Tatum & Reaume (53) and produced an adenine-dependent, methionine-independent mutant with pink colonies. Adenine-dependence was shown by Lindegren & Lindegren (4) to be the effect of a single gene; pink pigment is a correlated effect which depends on the synergistic effect of other genes as well, and only occurs in the presence of adequate amounts of methionine. Pink pigment is apparently produced following the interaction of a precursor of adenine and an excess of methionine plus other substances. Pigment is usually produced in organisms incapable of completing the synthesis of adenine and capable of producing a considerable amount of methionine. The variation in intensity of color in different pink organisms indicates that many other factors affect color intensity.

White, adenine-dependent variant colonies which retain their methionine-synthesizing ability often arise from pink cultures on vegetative propagation. Similar white segregants arising in the pink pedigree were subjected to genetical analysis. Hybrids of exceptional adenine-dependent, methionine-independent white cultures by pink cultures produced asci containing four pink cultures each, and asci from a hybrid of exceptional white by a standard adenine-independent, methionine-independent white produced two pink and two white cultures per ascus; the white cultures were methionine-dependent. Therefore, the inability of the exceptional adenine-dependent, methionine-independent cultures to produce the pink pigment was due to some mechanism which is restored to activity following hybridization.

This phenomenon was called a depletion mutation and the following hypothesis was invoked to explain the effect of outcrossing in restoring the pink color. Pink depends upon the presence of the

two genes ad and MET plus some other substances (X, Y, Z, etc.). The substance X is an essential component of gene X which has *n* other components besides X. Continuous production of pink exhausts the supply of X and results in the "running out" of the character. The stock to which the outcross is made carries gene X with an intact supply of the X component, for since the stock does not produce pink it does not exhaust its supply of the X substance. The outcross automatically restores the X substance and re-establishes the pink color. Other stocks may become white because Y or Z substances are exhausted. Mutations from pink to white result from the loss of some irreplaceable component easily supplied by outcrossing to any normal stock.

Nearly all haplophase yeast cultures, when plated on agar, produce a considerable variety of relatively stable colonial "mutants," while the corresponding diplophase cultures practically never produce colonial variants. Hybrids are produced by mixing the cells of two apparently unstable haplophase cultures together in a small amount of broth. After copulations occur, diploid cells are produced which subsequently sporulate. Genetical analyses of the hybrid are made by dissecting four-spored asci at random after sporulation has occurred. One of the puzzling things about this process is the fact that in spite of the apparently considerable mutational potential of the haplophase cultures, the tetrad analyses usually yielded surprisingly regular results. This was supposed at first to be the result of statistical sampling; in the enormous population of cells, the chances that a given zygote had been derived from the two preponderant genotypes is presumably very great. However, the demonstration of depletion mutation suggests that many of the variants which appear on the plates are depletion mutations which cannot be transmitted through the zygote. The reliability of the method of mass mating is greater than the variability of the haplophase would lead one to believe. Numerous trials have confirmed the reliability of the mass mating method, although at the time, it seemed curious that such apparently unstable gametes could produce such surprisingly stable zygotes. The stability of the diplophase, therefore, may not only be due to the fact that the dominant allele can "conceal" the recessive mutant, but two chromosomes may be able to produce a sufficient amount of genetic substance actually to prevent the occurrence of mutations.

NEUROSPORA

Shear & Dodge (32) named the genus *Neurospora* which contains both four-spored and eight-spored species. Dodge (33) discovered that the eight-spored species were heterothallic, while the four-spored species produced homothallic spores, due to the inclusion of one nucleus of each mating type in the spore.

The perithecium of *Neurospora crassa* produces a large number of asci, each containing eight spores. The spores fall into two categories with regard to mating type. Each spore gives rise to a thallus which produces a mycelium, on which male and female sex organs, as well as asexual conidia, are developed. The female sex organ is called a bulbil and contains an oogonium and trichogyne. The male sex organ produces sperm called spermatia. The spermatia from an A type thallus are incapable of fertilizing the oogonium developed on the thallus of the same type, but will cross-fertilize an oogonium of a complementary mating type. The superimposition of sex organs and mating type shows that mating type genes are self-sterility alleles and bear no relation to either sex organs or the evolution of sex.

Single ascospore cultures are characterized either on morphological or biochemical characters and hybrids are made either by transferring spermatia from a tube of one mating type to a tube of the complementary mating type, or by inoculating a single tube with conidia from each culture and allowing them to grow together. Lindegren & Lindegren (34) analyzed crossing-over in four regions marked by five loci bridging the centromere of *N. crassa*. They concluded that crossing-over was a nonrandom process; that local regions of the chromosome had specific patterns controlling the types of exchanges which occurred. This was not in agreement with the findings of Beadle & Emerson (35) in *Drosophila*. It was suggested that they may have studied regions too long to permit the detection of all possible exchanges. The stocks of *Neurospora* now available should make a reinvestigation of this problem a simple matter.

The treatment of *Neurospora* with ultraviolet and x-rays has been used for the production of mutations. Lindegren & Lindegren (36) treated individual spermatia and found that ultraviolet produced very few chromosomal aberrations, while x-rays produced a large proportion. Many of the variants reverted on subculture, but nearly one third of the ultraviolet variants were stable on sub-

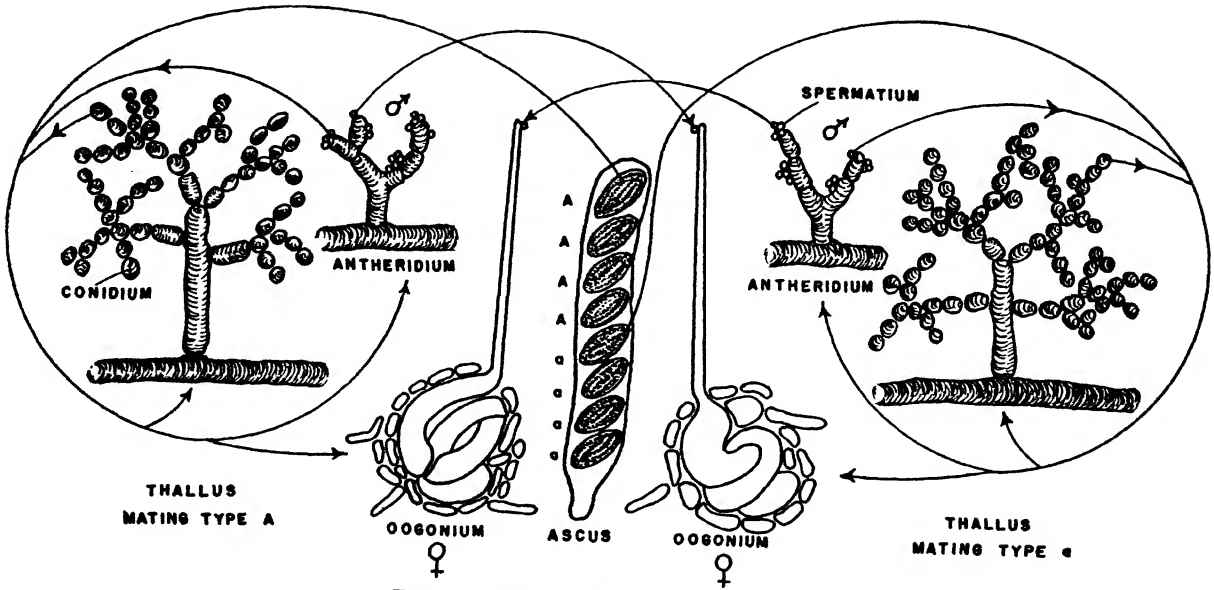

FIG. 5.—LIFE CYCLE OF *Neurospora crassa*.

culture, but produced only normal progeny when outcrossed. Therefore, the function which had been destroyed by treatment was restored in the heterozygous condition. At the time this was interpreted as a manifestation of cytoplasmic inheritance, but now the simpler concept of depletion mutation seems preferable. An extensive study of the effects of monochromatic radiation of *Neurospora* spermatia by Sansome, Hollaender & Demerec (37) has confirmed these results.

Beadle & Tatum (38) irradiated *Neurospora* conidia and fertilized complementary thalli with irradiated conidia and isolated the asci subsequently developed. When an individual ascus produced four mutant and four normal cultures, the mutant cultures were further analyzed for their ability to synthesize vitamins. This work in biochemical genetics has been summarized by Beadle (39) and has led to a great interest in the use of *Neurospora* for the analysis of biochemical mechanisms. It does not involve any discrepancy with Lindegren & Lindegren's finding of depletion mutations following radiation, since Beadle's mutants were only isolated after hybridization, at which time all depletions would have been restored.

Beadle and his co-workers have used *Neurospora* for the study of biochemical syntheses by selecting stocks differing in ability to produce enzymes which control steps in biochemical syntheses. Beadle and his group have proposed the one-gene-one-enzyme hypothesis which states that each gene synthesizes a specific and different enzyme. Delbrueck (40) has pointed out, that since no test has been devised by which this hypothesis could be disproved, additional evidence supporting it is not so much to be desired as the development of a critical test of its validity.

Lindegren (41) showed that one of the natural devices for preserving variation in *Neurospora* and presumably in other fungi is the formation of heterocaryons. Most of the fungi are multinucleate ceonocytes without cross walls in the hyphae, so that all the nuclei are commingled in a common cytoplasm, enabling the fungus to maintain great genetic variability. In nature, the heterocaryons are usually composed of nuclei of complementary mating type, with superimposed sterility genes which minimize or prevent the formation of perithecia until fertilization by another stock occurs. Beadle & Coonradt (42) pointed out the advantage of testing different mutants for allelism by making heterocaryons and observing whether or not the wild type is reconstituted. Ryan (43)

exploited the differences in rate of growth between mutants and heterocaryons as a means of testing for allelism. It should be pointed out, however, that neither the heterocaryon test for allelism nor the standard genetical test constitute proof of allelism. This problem is a fundamental problem of genetics. The same objections which Delbrueck (40) makes to the one-gene-one-enzyme hypothesis apply to assuming the existence of multiple allelic series. Thus far, no test has been devised which has established with certainty whether two mutants can be produced by changes at a single locus, and there are reasons for suspecting that multiple allelism does not exist.

VENTURIA

The brightest spot in the attempts to exploit the genetics of the fungi in solving the problem of pathogenesis is *Venturia*. The difficulties in solving problems of this type with the rusts have been pointed out. The smuts are better material than the rusts, but the fact that the parasite is a dicaryon and an obligate parasite is a limiting factor. *Venturia* is the most favorable organism in which the relation of a parasite to its host can be studied. It is an eight-spored ascomycete which produces haploid spores, the haplophase mycelium constituting the parasitic phase on the apple. The haplophase grows well on ordinary media and there is no complicating dicaryophase in the life cycle. The ability to act as a parasite must depend on some nutrient which the host produces that is essential to the parasite, or some relation between metabolic rates of host and parasite, and the extensive work on the nutrition of the ascomycetes suggest that relations of this type may be rather easily worked out. *Venturia* genetics has been attacked by Keitt (44) and his co-workers. There are many varieties of apples on which this parasite is pathogenic, and the pathogenicity of the different genotypes of *Venturia* is highly specialized and clearly under genetical control. It is the ideal situation for an analysis of the interplay of host and parasite and the genetical and nutritional interaction of two organisms bound together by this relationship. *Venturia* is a typical ascomycete, whose life cycle is practically identical with that of *Neurospora*, except that the haplophase is pathogenic to various apples. However, the genes controlling pathogenicity segregate regularly in the ascus and precise ratios are obtained.

In the analysis of a selected stock Keitt has shown that one gene controls parasitism on Haralson and Wealthy apples, while cultures carrying its allele are not pathogenic against these varieties, but are pathogenic for McIntosh and Yellow Transparent,

suggesting that gene A controls parasitism on the first two varieties, and its allele, gene a, controls parasitism on the other two. This situation only applies to the specific inbred stock of *Venturia* reported on, for earlier papers showed that the same gene did not control parasitism against these two varieties in all *Venturia* cultures. These indications suggest that study of the genetics of this fungus, especially the nutritional and vitamin deficiencies involved in the pathogenic stocks, give great promise of solving many problems involved in host-parasite relationship.

Glomerella

Glomerella is an ascomycete similar to *Neurospora*, except that it produces homothallic haploid spores. The fungus has been studied quite thoroughly by Edgerton, Chilton & Lucas (45), by Hüttig (46), and by Andes (47). They have all encountered the homothallic light cultures which revert to the self-sterile dark stocks incapable of producing large quantities of fertile perithecia from a single ascospore culture. The homothallic light stock ordinarily produces asci containing four light and four dark progeny, or eight dark progeny. All the asci in a given perithecium contain either four light or four dark or eight dark cultures. Lindegren (48) has shown that in *Neurospora* all the asci in a single perithecium (when matings are made as they are in the *Glomerella* work) arise from a single pair of nuclei associated at the initiation of the perithecium. On this fact, the work in *Glomerella* can be interpreted in terms of mutation from homothallic light to dark, and the association either of two dark mutant nuclei or one light and one dark nucleus at the initiation of the perithecium. The homothallic light mutates to dark and two kinds of perithecia are produced, the light/dark, or dark/dark. Mutation occurs so frequently that the light/light combination is only rarely found.

Penicillium

The discovery of penicillin has resulted in considerable interest in variations among species of *Penicillium* in penicillin production. Derx (49) proved by mating single ascospore cultures isolated from *Penicillium luteum* that this species is heterothallic. *Penicillium notatum*, from which penicillin is currently produced, is a form species rather than a true species. It probably originated from *Penicillium* ascospores and is perpetuated in nature asexually. The existence of heterothallic species in *Penicillium* suggest that the sexual mechanism may be exploited to obtain penicillin-producing strains.

Selective Agents

Geneticists have generally regarded the work of Avery and his collaborators (15) as convincing evidence that directed mutation from rough to Type III smooth pneumococcus can be achieved by treatment with a substance derived from the Type III organism. In spite of the fact that the method involved several special requirements for success, these seemed inherent to the case involved rather than factors which would invalidate the conclusions. Recent work has suggested that these special requirements may need more critical scrutiny. It has long been known that green bananas contain a substance which will prevent the transformation of virulent into avirulent pneumococci. Braun (57) has shown that globulin of susceptible genera of mammals contains a selective agent which prevents the transformation of virulent to avirulent *Brucella* and that pyrophosphate will achieve this same effect. The absence of this substance in resistant genera suggests one possibility: they may be naturally "immune" because when infected with virulent forms, the transformation to avirulent is not prevented and the avirulent organisms suffice to immunize the host against the virulent. Rogosa (58) has discovered a globulin factor in whey which prevents the transformation from smooth to rough in *Lactobacillus casei*. Mellon, Axelrod & McIlroy (59) have shown that acetate will prevent the transformation of virulent mucoid streptococcus into the smooth avirulent form.

A very few virulent Type III pneumococci are known to be capable of killing a susceptible mouse; a large number of rough pneumococci are not able to cause death. This is the current basis for assuming that no Type III pneumococci are present among rough organisms. The work on selective agents suggests the necessity for making synthetic mixtures of rough and virulent pneumococci to determine if a large population of rough may protect the mouse either mechanically or immunologically against a minute fraction of virulent organisms in the total population. If this be the case, Avery's transforming principle is merely another selective agent and we must assume that a small fraction of Type III organisms is present in all rough cultures capable of transformation.

In this unhappy event, the lack of a similar criterion for testing the colon bacillus transformation means that more refined methods will be needed here also.

The work on "directed" mutations has led many workers to

anticipate a high degree of control of mutation and the eventual production of "new" and "progressive" mutations—an objective not yet attained. Emerson (60) described an apparently new form of *Neurospora* which required sulphanilamide, but was poisoned by PAB. Zalokar (61) has recently shown that this was not a "new" character but an increase in susceptibility to PAB such that an antagonist was necessary to prevent the death of the organism from the PAB which it synthesized itself. Miller & Bohnhoff's production (7) of meningococci requiring streptomycin may be assumed to be a similar phenomenon.

Guthrie (62) has discovered an organism which produces more than 99 per cent mutants on irradiation. However, bacterial variants cannot be called true mutants until it can be demonstrated that they are not depletion mutations (see Saccharomyces).

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BACTERIAL METABOLISM[1]

By I. C. Gunsalus[2]

Laboratory of Bacteriology, College of Agriculture
Cornell University, Ithaca, New York,

This review will be limited largely to papers which emphasize the chemical aspects of bacterial metabolism and which have appeared during the past year. However, there is bound to be a certain amount of overlapping with enzyme studies and with growth factor and nutrition studies in relation to metabolic function. Because of space limitation and since adequate articles on growth factors are available, this phase of metabolism will be omitted unless the factors concerned deal specifically with metabolic function. Also omitted is any consideration of antibiotics and related metabolic products unless they deal with enzymes directly.

The past year has witnessed the continued development of several lines of research as well as the advent of a few new ones. Perhaps of major interest, as indicated both by the number and quality of contributions, are the studies on the function of essential metabolites. Highly significant additions have also been made to the knowledge of enzyme mechanisms, specifically in the enzyme-substrate studies with transglucosidase (1) and in studies of the mechanism of amino acid formation and degradation. On the problem of amino acid assimilation, the studies of Gale and his group (2) and of Hotchkiss (3) should be noted. In addition, the mechanism of biological protein synthesis, a problem not unrelated to the assimilation of amino acids by bacterial cells, has received attention and promising advances have been made. Microbiological studies are also contributing much to the knowledge of the mechanism of enzyme formation, especially with regard to adaptive enzymes (4).

In addition to the enzyme methods for approaching the synthetic and degradative processes of microorganisms, the competitive inhibitor method, used so successfully by Shive & Macow (5) and others to predict the function of essential metabolites, should

[1] This review covers the period from November 1946 to December 1947.

[2] Present address: Department of Bacteriology, Indiana University, Bloomington, Indiana.

be considered. The studies of Lampen & Jones (6) present further evidence as to the sequence of reactions of metabolic processes.

A most significant trend is to be noted in the number of bacteriologists and biochemists who are turning to bacterial enzymes as means of studying fundamental biochemical problems. As this year closes, one has the feeling that an approach is being made to the metabolism of amino acids, their mode of synthesis, and the synthesis of proteins in a fashion not unlike that which characterized the study of carbohydrate metabolism a decade or less ago.

It is assumed that the reader is familiar with the new *Annual Review of Microbiology* as well as *Advances in Enzymology*, *Vitamins and Hormones*, *Carbohydrate Chemistry*, and *Proteins and Enzymes*, which contain reviews on specific phases of microbial metabolism. A symposium on heredity and variation in microorganisms [*Cold Spring Harbor Symposium*, 11 (1946)] includes reports of metabolic studies involving mutants of *Neurospora* (7) and bacteria (8), as well as articles on the influence of environment on metabolism (9, 10) and of the factors controlling enzyme formation (4).

Kluyver & Custers (11) have abstracted the papers on bacterial metabolism which appeared in the Netherlands journals during the war years. A series of papers in honor of Dr. Kluyver's twenty-fifth year as professor of microbiology at Delft by his students and colleagues appears in Volume 12 of *Antonie van Leeuwenhoek Journal of Microbiology and Serology* (12). A book on chemical activities of bacteria by Gale (13), not as yet available to the author, has just appeared.

Oxidation

Several new aids to respiration studies have been suggested. Dorrell & Knight (14) have performed respiration studies with fungus homogenates. Endogenous respiration, which is usually high with mold preparations, may be decreased by aging the homogenates at a low temperature or by growing the cultures on carbohydrate-poor media. Stanier (15) suggested a method for determining the route of oxidation of various substrates based on multiple simultaneous adaptation and has employed the method to study the oxidation of a series of aromatic compounds. According to this hypothesis, cells grown on a given substrate should attack all intermediate compounds in the reaction series, whereas growth on a compound intermediate in the series should cause

adaptation to all subsequent members of the series. With the compounds studied, resting cells could also be adapted by incubation with the substrates for periods of twenty to sixty minutes.

Keilin & Hartree (16) have studied the cytochrome content of vegetative cells and spores of *Bacillus subtilis*. The absorption bands for cytochromes-*a*, -*b*, and -*c* were present in vegetative cells, and their respiration was sensitive to the usual inhibitors for iron systems. The spores exhibited slight respiration and showed only about 6 per cent as much cytochrome as the vegetative cells; the spores do, however, contain about 50 per cent as much hematin as the vegetative cells. The suggestion was made that this may serve as a precursor of the cytochromes.

Dole, Hawkings & Barker (17) have studied the fractionation of oxygen isotopes by growing bacterial cultures and found a very slight preferential use of oxygen 16 over oxygen 18. Whelton & Phaff (18) prepared a stable nonoxidative variant of *Saccharomyces cerevisiae* by the use of ethylene oxide.

Bellamy & Klimek (19) have reported the metabolic characteristics of a penicillin-resistant strain of *Staphylococcus aureus*. A strain with a sixty thousandfold increase in penicillin resistance was gram negative and grew only aerobically, also more slowly than the parent strain. Organisms such as *Streptococcus faecalis* and *Clostridium perfringens* which lack aerobic mechanisms did not develop penicillin resistance; this led to the suggestion that ability to develop penicillin resistance is associated with aerobiosis. [For the effect of penicillin on assimilation, see Gale (113).]

Organic acid oxidation.—Lominski *et al.* (20) studied the utilization of citric acid by *Escherichia coli*, which is not generally considered to be a citrate-utilizing organism. If the organism were grown in a peptone-citrate medium, adaptation to citrate oxidation occurred. *Staphylococcus aureus* was found not to use this substrate.

Kalnitsky & Barron (21) utilized fluoroacetate as an inhibitor of oxidative processes in various organisms, among them *Escherichia coli* and *Corynebacterium creatinovorans;* with the latter organism fluoroacetate, at a concentration of 0.002 *M*, was a very effective inhibitor of acetate, succinate, or malate oxidation. The oxidation of other compounds structurally similar to fluoroacetate such as glycine, glycollic, and glyoxalic acids was also markedly inhibited. Pyruvate oxidation by *Escherichia coli* and the gonococcus were inhibited to a smaller extent. Fluoroacetate is considered

to compete for enzyme with acetate and related compounds because of the similar bond distances of fluorine and hydrogen. Randles & Birkeland (22) reported that malonate inhibits methylene blue reduction with succinate as substrate by *Pseudomonas aeruginosa* but not by *Escherichia coli*. If *E. coli* was grown under aerobic conditions in the presence of acetate, the rate of methylene blue reduction with a series of organic acids, including acetate, succinate, fumarate, and malate, was greatly increased, thus indicating that these may mediate in the oxidation of acetate by this organism.

Lwoff *et al.* (23, 24) have presented evidence that a mutant of *Moraxella lwoffi* oxidized succinate, fumarate, and malate by a mechanism independent of the usual route through oxaloacetate followed by decarboxylation to pyruvate and carbon dioxide. It is suggested that pyruvate may be formed directly from malate without oxaloacetate serving as an intermediate. The triphosphopyridine nucleotide-linked malic acid decarboxylase of pigeon liver described by Ochoa *et al.* (25) answers this description. Ajl, White & Werkman (26) have shown that a number of C_4 dicarboxylic acids can fulfill the carbon dioxide requirement of heterotrophic organisms especially *Aerobacter aerogenes* and *Escherichia coli*, thus indicating the mediation of these compounds in the metabolism of the organisms mentioned.

The oxidation processes of a saprophytic acid fast organism, *Mycobacterium phlei*, has been explored by Edson & Hunter (27). With resting cells a series of carbohydrates and organic acids was found to undergo oxidation through a cyanide-sensitive series of carriers. A stable lactate enzyme was obtained in acetone powders and was found to catalyze the oxidation as far as acetate and carbon dioxide (28). This enzyme is noncyanide sensitive and flavine-adenine dinucleotide linked. The oxidation of hydroxy acids by several acid fast strains was investigated by Roulet *et al.* (29).

Carbohydrates and alcohols.—The number of oxidative reactions catalyzed by members of the genus *Acetobacter* and the genus *Pseudomonas* has been extended. Stanier (30, 31) has shown that ethyl alcohol is oxidized by *Pseudomonas fluorescens* to yield as much as 50 to 70 per cent of the calculated amount of acetic acid, thus adding acetic acid formation to the series of similarities between the *Acetobacter* and *Pseudomonas* genera. *Pseudomonas*

strains are in general relatively more acid sensitive. The oxidation of pentoses as far as the corresponding *onic* acids by several *Pseudomonas* strains has been reported by Lockwood & Nelson (32).

Carter *et al.* (33) found the oxidation of *meso*-inositol by *Acetobacter suboxidans* to yield scyllo-*meso*-inosose, and upon further oxidation, a product which was not isolated, possibly di-keto-inositol.

Mickelson & Shideman (34) studied the oxidation of glycerol by *Escherichia coli.* Inorganic phosphate disappeared with the probable formation of glycerol phosphate, since α-glycerol phosphate was also oxidized. Both ATP and inorganic phosphate stimulated the rate of oxidation. The respiration is both cyanide and iodoacetate sensitive, therefore, resembling the yeast enzyme system.

Reese (35) has studied the conditions necessary for the growth of cellulose-decomposing bacteria and has shown that oxidation is necessary for growth of the organisms. The enhancement of oxidation by the addition of iron indicates the presence of typical aerobic mechanisms.

Aromatic compounds.—The oxidation of a series of aromatic compounds by *Pseudomonas fluorescens* has been studied by Stanier (15) with his multiple simultaneous adaptation method. Mandelic and benzoic acid oxidation appears to occur through a similar series of enzymes; the other acids tested, phenylacetic and *p*-hydroxybenzoic acid, did not adapt during the oxidation of the former and, therefore, do not appear to be intermediates in the oxidation chain. From the curves presented, it appears that these compounds undergo approximately one-third complete oxidation. Investigation of the cause for the later reaction would be interesting to investigate, as would the other intermediates in the oxidation.

The oxidation of phenol and benzoic acid by a series of soil organisms has been shown by Evans (36) to occur through the analogous dihydroxy compounds followed by ring cleavage. Complete oxidation did not occur since only four moles of carbon dioxide were released per mole of phenol oxidized. From the data one cannot differentiate between assimilation and the accumulation of intermediate products as the cause of the incomplete oxidation.

The oxidation of carcinogenic hydrocarbons by marine bacteria was reported by Sisler & ZoBell (37), but precise data are not available as yet.

Hydrogenases.—Stephenson (38) has reviewed several phases of the hydrogen transport problem, especially with relation to the hydrogenases. The hydrogenases of *Proteus vulgaris* have been studied by Farkas & Fischer (39), who determined the characteristics of the enzymes by the exchange reaction between hydrogen and deuterium as well as by the reduction of fumarate. Fumarate reduction with hydrogen was also studied with *Escherichia coli* (40). It was concluded that two enzymes exist—one for hydrogen activation, a second for the activation of fumarate. Back and coworkers (41) studied the hydrogenase of *Escherichia coli* with cell free extracts and found oxygen, methylene blue, fumarate, and nitrate to serve as suitable hydrogen acceptors.

Lascelles & Still (42) have studied the nitrate, nitrite, and hydroxylamine reduction systems of *Escherichia coli* in the presence of hydrogen and have concluded from the optimum pH, effect of inhibitors, etc., that the three are quite distinct. Pollock (43) studied the adaptive nature of the nitratase of *E. coli* and the influence of various hydrogen donors upon this system.

Aubel *et al.* (44) determined the effect of azide on the anaerobic evolution of hydrogen by *Escherichia coli* and found that 0.0005 *M* would inhibit hydrogen release from either glucose or pyruvate, whereas 0.001 *M* did not inhibit respiration on these substrates.

Oxidation by sulfate-reducing bacteria.—The oxidation of hydrocarbons (45) and even numbered carbon fatty acids (46) by the sulfate-reducing bacteria has been reported by Rosenfeld. In the presence of reduced methylene blue as hydrogen donor, fatty acids were reduced by this organism. Butlin & Adams (47) have demonstrated that the sulfate-reducing organism, *Vibrio desulfuricans*, can grow autotrophically in the presence of hydrogen as an energy source.

Fermentation

Meyerhof (48) has reviewed his recent studies on the kinetics of cell-free alcoholic fermentation. These include the mechanism of the arsenate effect, the evidence for the existence of a 1,3-diphospho glyceraldehyde complex, and the relationship of adenosinetri-

phosphatase to the Harden-Young equation for hexosediphosphate accumulation.

Bacilli.—The study of the butylene glycol fermentation of aerobic bacilli which was begun during the war has been continued. *Bacillus subtilis*, both the Lawrence & Ford and the Marburg types, produce good yields of 2,3-butylene glycol, acetylmethylcarbinol, and glycerol aerobically (49). The former also produced solvents anaerobically, but the Marburg type did not grow under these conditions. The following equation has been suggested to describe the route of 80 to 90 per cent of the glucose fermented with selected strains.

$$3 \text{ glucose} \rightarrow 2(2,3\text{-butylene glycol}) + 2 \text{ glycerol} + 4\ CO_2 \qquad (a)$$

With many strains, however, the butylene glycol and glycerol yields fell below these levels. Small amounts of formic, acetic, and lactic acids and traces of ethyl alcohol were also formed. The butylene glycol formed consists of about two thirds of the *meso* isomer and one third of the *laevo*, whereas the lactic acid formed is largely the *l* (+) isomer.

The influence of aeration and of potassium and nitrogen sources on butylene glycol production by *Bacillus polymyxa* has been studied (50, 51, 52). With aeration, the yield of butylene glycol is decreased with a corresponding increase in acetylmethylcarbinol formation; the sum of the two equalled about one-half mole per mole of glucose fermented (50).

Colon-aerogenes bacteria.—The fermentation products of *Serratia marcescens* (aerogenes type gram negative rod) include butylene glycol in yields as high as one-half mole per mole of glucose fermented (53), the other products being largely lactic and formic acids, ethyl alcohol and carbon dioxide. Aerobically, the yield of carbon dioxide is increased at the expense of these compounds without markedly affecting the yield of butylene glycol. As is usual for the gram negative rods, *d*(−) lactic acid is formed.

Factors affecting butylene glycol production by *Aerobacter aerogenes* have been studied by Freeman (54). In addition, Paretsky & Werkman (55) have shown aeration and methylene blue to favor the conversion of butylene glycol to acetylmethylcarbinol.

Genus Clostridium (butyric-butanol fermentation).—Simon (56) prepared resting cells and acetone-dried preparations of *Clos-*

tridium acetobutylicum with which he determined fermentability and the products from a series of substrates. Among the hexose sugars, sugar acids, and sugar esters, alteration of the first carbon did not affect the fermentation nor the yield of butyric acid, whereas alteration of the sixth position resulted either in nonfermentation or in a decreased yield of butyric acid. The fermentation of hexosediphosphate led to methyl glyoxal and pyruvate rather than to the normal products, whereas pyruvate fermentation proceeded only as far as acetate; i.e., it did not result in butyrate formation.

Further studies on fermentation in the presence of carbon monoxide (57) confirmed the formation of lactic acid as reported by Kempner (58). In addition, Simon showed that pyruvate breakdown was inhibited by carbon monoxide, thus suggesting that the pyruvate oxidase or an enzyme for a closely related subsequent step may be an iron-containing enzyme.

Barker (59) has discussed the characteristics of *Clostridium kluyveri* and the reactions which lead to the formation of the longer chain fatty acids with this culture.

Bhat & Barker (60) have isolated a new anaerobe, *Clostridium lacto-acetophilum*, capable of utilizing lactate as a carbon and energy source. Enrichment cultures grew well on lactate medium, but pure cultures required in addition the presence of acetate, biotin, *p*-aminobenzoic acid, and traces of yeast autolysate for growth. Lactate is broken down by the following suggested reactions:

$$\text{lactate} \rightarrow \text{acetate} + CO_2 + 4H \qquad (a)$$

$$4H \rightarrow 2H_2 \qquad (b)$$

$$2\ \text{acetate} + 4H \rightarrow \text{butyrate} + 2H_2O \qquad (c)$$

The excess of acetate required in reaction (*c*) over reaction (*a*) indicates the function of acetate as an external hydrogen acceptor; otherwise, the excess of reduced product, hydrogen, stops the fermentation. During the fermentation of pyruvate,

$$\text{pyruvate} \rightarrow \text{acetate} + CO_2 + 2H \qquad (d)$$

fermentation proceeds without the addition of external hydrogen acceptor since the acetate and hydrogen are in the proportions required for reaction (*c*). Although growth with glucose as an energy source can occur quite normally with the release of carbon

dioxide and hydrogen, energy release from lactate apparently cannot occur without an external hydrogen acceptor. From the data the authors also suggest a relationship between the quantity of butyrate produced and the residual acetate; this may be conditioned by an equilibrium involving these compounds or may possibly be conditioned by the hydrogen pressure. The question whether other anaerobes may also utilize lactate as an energy source in the presence of suitable hydrogen acceptors is considered.

Barker & Elsden (61) have reported the utilization of carbon dioxide in the formation of acetic acid and glycine by *Clostridium cylindrosporus*. Anaerobic fermentation of uric acid yields carbon dioxide, ammonia, glycine, and acetate. If the fermentation occurs in the presence of isotopically labeled carbon dioxide, the C^{14} appears in the methyl and carboxyl carbons of acetate and in the carboxyl carbon of glycine. Glycine and acetate appear to be formed by different mechanisms and are not interconvertible.

Aubel and co-workers (62) have studied the effect of oxygen on the oxidation-reduction potential and on the growth of cultures of the anaerobic organisms, *Clostridium saccharobutyricum* and *Clostridium sporogenes*. Aeration in the presence of a small number of cells prevented growth but not fermentation. Hydrogen peroxide (63) in low concentrations caused a reversible inhibition of fermentation; larger amounts stopped fermentation entirely.

Tytell & Tytell (64) reported upon the conditions influencing glucose dehydrogenase production by *Clostridium perfringens*. Prévot & Enescu (65) studied nitrate reduction by this organism using methylene blue as a hydrogen acceptor. They also studied the fermentation products of *Clostridium corallinum* (66) and the diversion of the fermentation of *Clostridium bifermentans* and *Clostridium caproicum* (67).

Amino acid fermentation (propionic and acetic).—Two new amino acid-fermenting organisms have been isolated by Cardon & Barker (68, 69) using enrichment techniques. One of these, *Clostridium propionicum*, formed propionic acid from alanine and related compounds, whereas the second, *Diplococcus glycinophilus*, utilized only glycine as an energy source.

Fermentation balances, with resting cell suspensions of *Clostridium propionicum*, yielded two types of fermentation depending upon the state of oxidation of the substrates used. These may be written as follows:

$$\left.\begin{matrix}3\text{ alanine}+2H_2O\\ 3\text{ lactate}+\text{——}\\ 3\text{ acrylate}+2H_2O\end{matrix}\right\}\rightarrow 2\text{ propionate}+1\text{ acetate}+1\ CO_2+\left\{\begin{matrix}3NH_3\\ H_2O\\ \text{——}\end{matrix}\right. \quad (a)$$

$$\left.\begin{matrix}3\text{ serine}+H_2O\\ 3\text{ pyruvate}+H_2O\end{matrix}\right\}\rightarrow 1\text{ propionate}+2\text{ acetate}+2\ CO_2+\left\{\begin{matrix}3NH_3\\ \text{——}\end{matrix}\right. \quad (b)$$

A third reaction, analogous to (*b*) except for the chain length of substrate, occurs with threonine:

$$3\text{ threonine}+H_2O\rightarrow 1\text{ butyrate}+2\text{ propionate}+2\ CO_2+3NH_3$$

A number of other amino acids are also attacked including glutamic, alanine, valine, leucine, phenylalanine, cystine, glycine, and, more slowly, histidine. This organism is not a true propionic acid bacterium but apparently carries out this type of fermentation in response to the oxidation-reduction state and the chain length of the acids attacked. It should also be noted that the fermentation of lactate by this organism differs from that which occurs with *Clostridium lacto-acetophilum* described in the previous section.

The second amino acid fermenting organism, *Diplococcus glycinophilus* (68, 69), utilizes only glycine as an energy source; this is broken down according to the following equation:

$$4\text{ glycine}+2H_2O\rightarrow 3\text{ acetate}+2CO_2+4NH_3$$

In the presence of glycine, pyruvate and serine are also broken down slowly. If fermentation occurs under conditions in which the gas pressure is relieved (decreased hydrogen pressure), hydrogen and carbon dioxide are produced with a decrease in acetate yield. The relationship of this amino acid fermentation to the reactions of other anaerobes in which glycine serves either as a hydrogen acceptor or donor is discussed.

Methane fermentation.—Kluyver & Schnellen (70) studied the fermentation of carbon monoxide by methane bacteria using *Methanosarcina barkeri* and reported the following reaction to occur:

$$4CO+2H_2O\rightarrow 3CO_2+CH_4 \quad (a)$$

This reaction has been shown to occur by route of the two following reactions:

$$4CO+4H_2O\rightarrow 4CO_2+4H_2 \quad (b)$$
$$CO_2+4H_2\rightarrow CH_4+2H_2O \quad (c)$$

Reaction (*b*) has been demonstrated by trapping the carbon dioxide with alkali, in which case, the calculated amount of hydrogen

was found. Reaction (*c*) was demonstrated by methane production in the presence of hydrogen and carbon dioxide. In the presence of a hydrogen atmosphere, all the carbon monoxide may be converted to methane. *Methanobacterium omelianskii* does not utilize carbon monoxide and hydrogen; furthermore, in the presence of carbon dioxide, hydrogen, and carbon monoxide, methane is formed without carbon monoxide being used. Therefore, carbon monoxide cannot be an intermediate in the formation of methane from carbon dioxide.

Lactic acid bacteria and Staphylococci.—Two more reactions of pyruvate have been shown to be reversible. Wikén and co-workers (71) have shown by an exchange reaction with isotopic carbon dioxide that the dismutation reaction in this organism is reversible. Using a cell-free preparation in the presence of pyruvate, inorganic phosphate, and isotopically labeled carbon dioxide, the isotope was found in the carboxyl group of pyruvate. Thus it is suggested that the reaction, 2 pyruvate+phosphate $\rightleftarrows$ 1 lactate +1 acetate (acetylphosphate)+1 CO_2, is reversible.

Watt & Krampitz (72) have also shown that isotopic carbon is fixed in the carboxyl group of pyruvate during acetylmethylcarbinol formation by *Staphylococcus aureus*. These workers have postulated the occurrence of α-acetolactic acid as an intermediate in this reaction because this acid is decarboxylated rapidly with the formation of acetylmethylcarbinol. Manganese but not cocarboxylase is required. If this were the mechanism, the carboxylation would occur in the beta position in analogy to other known beta carboxylases, as oxaloacetate, which require manganese but not cocarboxylase.

Gross & Werkman (73) confirmed the mechanism of acetylmethylcarbinol formation by *Aerobacter*, yeast and muscle tissue as occurring by three mechanisms, namely: *Aerobacter* forms acetylmethylcarbinol from two moles of pyruvate and does not utilize acetaldehyde, the yeast and pig heart enzymes involve one mole of pyruvate and one of acetaldehyde. The pig heart enzyme also forms acetylmethylcarbinol from acetaldehyde alone, apparently without endogenous pyruvate utilization, since no carbon dioxide is released. Using yeast juices (74) and isotopically labeled acetaldehyde, these workers found heavy carbon distributed throughout the acetylmethylcarbinol molecule and suggested the possibility of a symmetrical intermediate.

Douglas (75) found hydrogen peroxide to be utilized in the metabolism of *Lactobacillus brevis*. Lactic acid or glucose is oxidized to acetate plus carbon dioxide without the accumulation of peroxide. Furthermore, under anaerobic conditions, hydrogen peroxide served as a hydrogen acceptor for the oxidation of lactate according to the following reaction:

$$\text{lactate} + 2H_2O_2 \rightarrow \text{acetate} + CO_2 + 3H_2O$$

The cells did not activate lactate or peroxide alone nor did heat-killed cells bring about the destruction of peroxide, indicating that a peroxidase is involved. Douglas suggests that this enzyme is comparable to the noncyanide sensitive peroxidase enzyme of *Streptococcus mastiditis* previously reported by Greisen *et al.* (76).

Gunsalus (77) has reported the products of anaerobic glycerol fermentation by *Streptococcus faecalis*. In analogy to the reaction described by Bhat & Barker (60) for *Clostridium lacto-acetophilum*, this organism is unable to ferment glycerol in the absence of an external hydrogen acceptor. The general equation for the fermentation of glycerol by streptococci is:

$$\text{glycerol} \rightarrow \text{lactate} + 2H$$

The strain studied can utilize fumarate as a hydrogen acceptor according to the following reaction:

$$\text{glycerol} + \text{fumarate} \rightarrow \text{lactate} + \text{succinate}$$

In the presence of excess fumarate, products more oxidized than lactate, i.e., acetate and carbon dioxide, are formed.

Hoff-Jørgensen, Williams & Snell (78) reported the preferential use of lactose by a strain of *Lactobacillus bulgaricus*. Their data were similar to those for the preferential utilization of disaccharides by other organisms (79, 80), except that this appears to constitute a limiting case in which monosaccharides are not utilized at all in the absence of the disaccharide.

Stimulation of the glycolytic rate of various lactic acid bacteria has been reported. In addition to McIlwain's (81) observation that glutamine stimulates the glycolytic rate, Foust & Gunsalus (82) have shown that glutamic acid, histidine, and ammonia will stimulate the rate of glycolysis of washed suspensions of *Streptococcus faecalis* as much as four- to fivefold. Woolley (83) has also reported that strepogenin will stimulate the glycolysis of washed cells of *Lactobacillus casei*. As yet the position or mode of action of these

effects is unknown. However, their relationship to the assimilation studies of Hotchkiss (3) and Gale (2) should be considered.

Tsuchiya & Halvorson (84) have reported upon the conditions necessary for the preparation of glycolytically active washed cells of lactobacilli; the resting cells could be lyophilyzed without great loss in activity. Halvorson & Muedeking (85) found greater fermentation rates of *Lactobacillus casei* on yeast extract media than on the best synthetic medium obtainable, but were unable to determine the cause for these differences.

Bacterial Enzymes

Spiegelman (86) has discussed in some detail the factors involved in enzyme formation.

Disaccharide formation.—Several recent reviews on the formation of polysaccharides by microorganisms should be noted: Stacey (87) on bacterial cellulose and dextrans, capsular polysaccharides, and other macromolecules; Evans & Hibbert (88) on bacterial polysaccharide formation.

Doudoroff, Barker & Hassid (89), continuing their studies with partially purified sucrose phosphorylase of *Pseudomonas saccharophila*, have made very significant progress. They present evidence to indicate that, in the absence of phosphate, sorbose replaces fructose in the formation of glucosido-sorboside from sucrose; furthermore, glucose-1-phosphate undergoes an exchange reaction with isotopic phosphate in the absence of fructose. The indicated mechanisms may be written as follows:

$$\text{sucrose} + \text{enzyme} \rightleftarrows \text{glucose-enzyme} + \text{fructose}$$

$$\text{glucose-1-phosphate} + \text{enzyme} \rightleftarrows \text{glucose-enzyme} + \text{phosphate}$$

Thus it appears that the enzyme can substitute a linkage for the analogous glucosido linkage in sucrose or in glucose-1-phosphate, thus adding substantial evidence to the enzyme substrate theory. These findings were interpreted as a glucose transport function, and the enzyme was renamed "transglucosidase." Two new disaccharides, one a nonreducing disaccharide and the second a reducing disaccharide have been formed with this enzyme (90); this is the first reducing disaccharide reported to be formed by the enzyme. Arsenate has been substituted for phosphate (91) and found to replace phosphate catalytically, but the arsenate compound breaks down spontaneously; this is analogous to the spontaneous break-

down, without energetic coupling, of phosphoarseno glyceric acid (48, 92). A review discussion of the enzymatically synthesized disaccharides is found in the Neuberg memorial number of the *Archives of Biochemistry* (93).

Cellulose formation.—Hehre, Carlson & Neill (94) have reported the formation of starch-like material from glucose-1-phosphate by a number of bacteria, including *Corynebacterium diphtheriae* and several hemolytic streptococci; these compounds gave a blue color with iodine.

Cellulose formation by growing and resting cells of the *Acetobacter* has been reported. Kaushal & Walker (95) demonstrated that the polysaccharide formed from a number of substrates was cellulose as indicated by x-ray diffraction studies. Hestrin *et al.* (96) have shown that resting cells of *Acetobacter xylinum* form cellulose under aerobic conditions in the presence of utilizable carbohydrates. Respiration was required for cellulose formation.

Crystalline enzymes.—The first of the bacterial enzymes has been crystallized. Meyer, Fuld & Bernfeld (97) reported the purification and crystallization of bacterial amylase from *Bacillus subtilis*. The enzyme is an α-amylase similar to that from pancreas.

Herbert & Pinsent (98) have crystallized bacterial catalase from *Micrococcus lysodeikticus*. This enzyme is freed from the cells by lysozyme, precipitated by alcohol, and finally crystallized from ammonium sulphate. The bacterial enzyme is similar to the catalase enzyme crystallized from horse, cow, and sheep blood. On the basis of the recoveries, it is estimated that the cells contain about 2 per cent catalase on the dry weight basis; molecular weight estimations indicate the presence of about twenty thousand molecules of catalase per cell.

Hydrolytic enzymes.—Zamecnik & Lipmann (99) have demonstrated that lecithin interferes with the combination of *Clostridium perfringens* antitoxin and the α-toxin, which involves a lecithinase. When the enzyme was first allowed to act on the lecithin the antitoxin failed to inhibit the lecithinase action, though it did gradually decrease the reaction rate. This inhibition is suggested as a possible factor in the lack of success of antitoxin treatment for advanced cases of *Clostridium perfringens* infections.

There have been reports on the production of various hydrolytic enzymes by bacterial cultures. LePage, Morgan & Campbell (100) have reported upon the conditions favoring the production

and purification of penicillinase, as have Housewright & Henry (101), who studied the kinetics and properties of this enzyme and reported that penicillinase is an adaptive enzyme produced only in response to the presence of penicillin (102).

Buck (103) has reported on the characteristics of a heat-resistant phosphatase from *Lactobacillus enzymothermophilus*. His work suggests that one factor involved in thermophilic behavior may be greater thermostability of the enzymes. The mechanism of this stability is, however, unknown.

Studies on hyaluronidase enzymes of the clostridia (104) and the streptococci (105) have been reported. The latter were shown to be adaptive.

Lipolytic enzymes of sulfate-reducing anaerobes and of *Clostridium perfringens* have been studied (106).

Site of action of metabolic inhibitors.—Krampitz & Werkman (107) reported inhibition of nucleic acid or nucleotide metabolism by penicillin. The data suggest that the nucleotide pentose is oxidized as follows:

$$(\text{pentose}) \rightarrow 1 \text{ acetate} + 3CO_2 + H_2O$$

The pentose level in the cells decreased during respiration; but, in the presence of penicillin, the respiration was inhibited and pentose did not disappear. Ribose and ribose-5-phosphate are not metabolized by intact cells, but ribonucleic acid is decomposed to the extent of 80 to 90 per cent in accordance with the reaction suggested above.

Geiger (108) has reported the relationship of the antibacterial action of quinones and hydroquinones to inactivation of the sulfhydryl groups of the enzymes. Gram negative organisms are usually affected less than gram positive organisms; with the gram negative cultures the addition of sulfhydryl compounds will also reactivate the enzymes.

Assimilation

Amino acids.—A beginning on the mechanism of amino acid assimilation by bacterial cells has been made by Hotchkiss (109), who showed with *Staphylococcus aureus* that, in the presence of an oxidizable substrate, amino acids were assimilated and converted to peptides. In the presence of glucose alone, oxidation occurred and inorganic phosphate was esterified, whereas the addition of

amino acids decreased the amount of inorganic phosphate taken up in relation to the number of acids added. During assimilation, the nitrogen content of the cells was increased as much as 25 per cent and in addition some polypeptides were liberated into the medium.

Gale (110) reported the assimilation of amino acids by resting cells of *Streptococcus faecalis*. Free lysine, glutamic acid, ornithine, and histidine were demonstrated to occur within the cells. An energy source is required for glutamate assimilation, but lysine enters the cell by diffusion; neither amino acid will diffuse out of the cell in the absence of an energy source. The concentrations of these amino acids within the cells as compared with the medium have been studied and relatively great differences in concentration have been found to exist across the cell membrane, differences as great as 500 to 800 μl. (22 to 36 μ moles) per ml. of the amino acids. Arginine will partially substitute for glucose as an energy source for the assimilation of glutamic acid. Tentative data indicated that aspartic acid behaves in a fashion similar to glutamic acid.

Further, Gale & Taylor (111) found a series of surface active agents, including tyrocidin and several detergents, which allow the loss of amino acids from the internal environment of the cell and have suggested that this leakage may account for the disinfectant action of the agents. Taylor (112) studied the assimilation of amino acids by several organisms and reported a correlation between gram reaction and the accumulation of amino acids by assimilation. The gram positive cocci accumulated glutamic acid, whereas gram negative rods did not.

Gale (113) has studied the influence of penicillin on the assimilation of various amino acids in *Staphylococcus aureus*, including penicillin-resistant strains of this organism. It was suggested that if assimilation were inhibited, the level of glutamic acid within the cell would decrease, whereas, if the metabolism of the cell were inhibited, the level of glutamate within the cell would increase. In the presence of penicillin, the level of the free glutamic acid within the cell decreased, thus indicating that assimilation was inhibited. In this case also it was suggested that failure of the cells to grow resulted from starvation due to a depletion of the amino acid level of the cells. A concentration of penicillin which will inhibit glutamic acid assimilation is comparable to that required to prevent growth.

The utilization of ammonia by *Serratia marcescens* was studied by McLean & Fisher (114), who showed with resting cells that the addition of ammonia increased the respiration rate and that the higher rate was maintained until the ammonia was utilized, approximately two moles of oxygen being required per mole of ammonia assimilated. Analysis of the suspending medium and the cells after the period of assimilation showed that the ammonia was incorporated into cellular material. Thus a gram negative organism capable of synthesizing amino acids used respiration energy to assimilate ammonia in a manner analogous to the assimilation of amino acids by gram positive organisms in the presence of an energy source (109, 110).

Assimilation of carbon compounds.—White & Werkman (115) studied the assimilation of labeled acetate by yeasts in an attempt to determine if the acetate was converted to fat and fatty acids. They found that isotopically labeled acetate added to growing cultures stimulated the storage of the isotope in fat; the fat content of the cells was approximately doubled in the presence of acetate. Only a trace of labeled carbon was found in lactate and this was in the carboxyl group. Isotopically labeled carbon dioxide was not converted to fat; thus, acetate is used directly and not via carbon dioxide. White, Krampitz & Werkman (116) have also studied the assimilation of acetate by yeasts.

Brockmann & Stier (117) have studied the effect of sodium azide on the fermentative ability of various yeasts. The addition of 0.002 *M* azide increased the rate of yeast fermentation and at the same time interfered with assimilation and the storage of energy-rich phosphate. These workers suggest that the influence of azide on fermentation is similar to that of arsenate.

The stimulation of bacteriophage formation by indole-3-acetic acid, as demonstrated by Cohen & Fowler (118, 119), may be another assimilatory process. However confirmation of the effect is still needed.

Amino Acid Synthesis and Breakdown

Although the nitrogen fixation problem will be reviewed by Virtanen in Volume II of the *Annual Review of Microbiology*, it may be pertinent to mention here that Burris & Wilson (120) have now reported the utilization of ammonia as an intermediate in nitrogen fixation and thus in amino acid synthesis by *Azotobacter*.

The utilization of ammonia by *Serratia marcescens* during endogenous respiration also appears to involve amino acid synthesis [McLean & Fisher (114)].

Freeland & Gale (121) have analyzed a number of bacteria and yeasts for amino acid content and have found that for those amino acids which may be determined by the decarboxylase method, the medium does not significantly affect the composition of the cells. The amino acid content of gram positive and gram negative organisms is similar with the possible exception of arginine, which comprises about 10 per cent of the total nitrogen of the gram negative rods and about half this level in gram positive cocci. Several bacilli were tested and found to lie midway between these in arginine content.

Synthesis of methionine and cystine.—Lampen, Jones and coworkers (122, 123) have contributed to the mechanism of synthesis of the sulfur-bearing amino acids by *Escherichia coli.* Most of these studies were carried out either with ultraviolet-produced mutants or with metabolic inhibitors. Their work showed that methionine can be replaced by its keto acid analogue or by homocysteine.

For *Escherichia coli*, methionine (123) could be partially replaced by norleucine; possible mechanisms were suggested (5). Other studies (124), employing mutants to determine the route of amino acid synthesis from sulfate, led to the suggestion that the sulfate is reduced to hydrogen sulfide, followed by the reversal of cysteine desulfurase to form cysteine. No data are available, however, on this point. Possible routes of synthesis, involving cysteine, cystathione, homocysteine, and methionine, were also presented. However, these suggestions must remain tentative until further data are available.

Lampen & Jones (125) also discussed the action of *p*-aminobenzoic acid and pteroylglutamic acid in the synthesis of methionine and lysine.

Horowitz (126) has used *Neurospora* mutants to study the synthesis of sulfur-containing amino acids. With these mutants it appeared that the route of synthesis is cysteine, cystathione, homocysteine, and methionine.

Methionine synthesis is inhibited by 2-chloro-*p*-aminobenzoic acid and by sulfanilamide; these inhibitors act at different points in the synthetic mechanism since strains resistant to 2-chloro-*p*-aminobenzoic acid were still sensitive to sulfanilamide, while cells

resistant to sulfanilamide were 2-chloro-*p*-aminobenzoic acid-resistant (127).

Interconversion of amino acids.—With *Torulopsis utilis*, C^{13} in the carboxyl group of alanine was released as respiratory carbon dioxide, whereas carboxyl-labeled glycine was converted to serine and proline as well as to respiratory carbon dioxide (128). Therefore, the formation of glycine from serine is reversible [Shemin (129)].

Tryptophane.—During the past year, two groups of workers have contributed to the studies on tryptophanase and have given some clarification to the mechanism of action of this enzyme. Dawes, Dawson & Happold (130), continuing their studies, have shown that about 25 per cent of the tryptophanase present in acetone-dried preparations may be obtained in a cell-free state. These extracts were inactivated by dialysis and reactivated by recombination of the enzyme and the dialyzing fluid. Further studies by these workers (131) showed the effect of a series of inhibitors on the enzyme action. In agreement with Tatum & Bonner (132), indole production was found to be inhibited by the presence of serine (133). Both serine and alanine were oxidized more rapidly by the enzyme system than was tryptophane. Evidence was obtained for the accumulation of alanine during tryptophanase action in the presence of mepacrine, to inhibit flavine-mediated respiration. The partially resolved enzyme was reactivated in the presence of pyridoxal phosphate, riboflavin, and diphosphopyridine nucleotide.

Wood, Gunsalus & Umbreit (134) isolated a cell-free tryptophanase from *Escherichia coli*. Their preparations catalyzed the breakdown of tryptophane according to the following reaction:

$$\text{tryptophane} \rightarrow \text{indole} + \text{pyruvate} + NH_3$$

The enzyme was completely resolved and found be to reactivated by pyridoxal phosphate. The system was somewhat sensitive to cyanide, possibly due to the carbonyl group of the coenzyme. As neither serine nor alanine was broken down by the cell-free enzyme preparation, they do not appear to be intermediates in the reaction.

Fildes & Rydon (135) have studied growth inhibition of *Bacterium typhosum* by a series of substituted tryptophane and indole derivatives.

Sources of amino acids for microorganisms.—Simmonds, Tatum

& Fruton have reported the utilization of a series of amino acid peptides by mutants of *Escherichia coli*. The leucineless mutant of *Escherichia coli*, obtained by x-radiation, has been shown to utilize a series of leucine di- and tri-peptides (136). Peptides which contained either free amino or carboxyl groups of leucine were used as was the penta-peptide in which both were bound; leucinamide and N-acetyl leucine were not active.

Utilization of phenylalanine and tyrosine was also studied (137), and, as in the case of leucine, the peptides replaced the respective amino acids for growth. Several of the peptides were less active on a molar basis than the free amino acids. Phenylpyruvic acid replaced phenylalanine, and *p*-hydroxyphenylpyruvic acid substituted for tyrosine, but the acetyl and dehydro derivatives were not utilized, nor were the D-amino acids active. D-Glutamic acid, as well as L-glutamic acid, is however active for the growth of *Lactobacillus arabinosus* [Dunn *et al.* (138)], both isomers having been found by analysis to be present in the cellular material. On the other hand, *Streptococcus faecalis* and *Leuconostoccitrovorum* did use the D-isomer of glutamic acid for growth.

Fruton and co-workers (139) have tested the ability of *Escherichia coli* to utilize various acetyl dehydroamino acids. It has been postulated that a condensation between acid amides and keto acids followed by reduction might occur as a route for peptide synthesis. Acetyl dehydrotyrosine was not used for growth, but in the presence of tyrosine this compound disappeared with the formation of a substance which has been isolated but not as yet completely identified.

Beadle and co-workers (140) have reported the existence of kynurenine as an intermediate in the formation of nicotinic acid from tryptophane by *Neurospora*.

Imbalance.—Studies on amino acid imbalance were presented by Shive *et al.* (141); β-2-thienylalanine inhibited tyrosine synthesis. Phenylalanine was converted to tyrosine, but tyrosine did not serve as a source of phenylalanine. It was suggested that tyrosine may be formed from phenylalanine by direct oxidation of the aromatic ring, and thus phenylpyruvic acid need not serve as an intermediate. High levels of tyrosine inhibited phenylalanine synthesis (142), very probably as a competitive inhibitor. Tryptophane inhibited the utilization of phenylalanine; the growth of

Streptococcus faecalis, for example, was inhibited by two to ten mg./ml. of DL-tryptophane and the inhibition was reversed by 40 to 100 μg. of phenylalanine. Thus tyrosine prevented synthesis, whereas tryptophane (143) appeared to compete for the enzymes responsible for phenylalanine utilization.

Other cases of imbalance were shown by the studies of Koser & Kasai (144), who found that high levels of nicotinic acid or nicotinamide inhibited growth. The inhibition could be relieved by yeast extract, but not by a vitamin mixture. The agent responsible for the reversal of the inhibition has not as yet been determined.

The Function of Essential Metabolites

Biotin.—At the close of the past year, it was known that biotin-deficient yeast showed a depressed rate of respiration and fermentation which could be relieved by biotin and ammonia (145) and that aspartic acid exerted a biotin-sparing action for *Torula cremoris* (146). It had also been suggested that biotin might mediate in the utilization of carbon dioxide (147).

During the present year, very important advances have been made in understanding the function of biotin. Stokes *et al.* (148 149) observed a relationship between biotin and aspartic acid. In the presence of excess biotin, aspartic acid could be eliminated from the medium for most lactic organisms commonly used for microbiological assay, but with limited levels of biotin (0.5×10^{-3} μg. per 10 ml.) aspartic acid was required for growth. Cells grown in the absence of aspartic acid synthesized this amino acid, as indicated by assays with *Leuconostoc mesenteroides*, which were unable to dispense with aspartic acid in the presence of biotin. Thus, biotin functions in the formation of aspartic acid. Following this lead, Stokes *et al.* could not find an altered rate of transamination in biotin deficiency. Subsequently Lardy, Potter & Elvehjem (150) studied the role of biotin in the growth of *Lactobacillus arabinosus* and observed that oxaloacetate would promote growth on a biotin- and aspartic acid-deficient medium and that bicarbonate would stimulate growth in a biotin-rich medium, thus indicating the function of biotin in the carbon dioxide fixation step. Other organic acids, including pyruvate, succinate, fumarate, malate, and α-keto glutarate, did not increase growth. In the same journal Shive & Rogers (151), using the competitive inhibitor approach,

reported a relief of biotin inhibition by α-keto glutarate—an observation which was interpreted as indicating that decreased biotin synthesis affected first α-keto glutarate formation.

Shive & Rogers cited a personal communication from Garrison & Eakin, which indicated that the function of biotin involves the formation of oxaloacetate from carbon dioxide and pyruvate during oxidation by yeast.

A new inhibitor of biotin synthesis (2-oxy-4-imidazolidine-caproic acid) (152) is reported by Rogers & Shive; since prevention of toxicity was competitive with desthiobiotin, but noncompetitive with biotin, it was suggested that the point of inhibition was between the desthiobiotin and biotin.

Lichstein & Umbreit (153) were also able to obtain evidence for the function of biotin in carbon dioxide fixation, using resting cell suspensions of *Escherichia coli*. A technique of aging cell suspensions in acid buffer was devised in which the cells were rendered deficient with regard to their ability to release carbon dioxide in a reaction involving a series of acids including aspartic, fumaric, and malic. A vitamin mixture, or biotin alone, reactivated the enzyme system. The release of carbon dioxide in the presence of a trapping agent for pyruvate led to the observation of the action of biotin in oxaloacetate decarboxylase. Thus the function of biotin in carbon dioxide fixation has been approached from both directions. Lichstein & Umbreit (154) have also suggested the function of biotin in deaminases for aspartic acid, serine, and threonine, based on increased rates of ammonia release. Whether this represents a separate function or merely an extension of the observation of the function in carbon dioxide fixation remains to be determined. Studies with biotin-deficient tissue by Ochoa *et al.* (155) have confirmed the function of biotin in the oxaloacetate decarboxylase of animal tissue, but purification of this enzyme has failed to reveal different biotin levels in the enzyme from deficient and normal tissues.

Axelrod, Hofmann and co-workers (156) have continued studies on the metabolism of oxybiotin by yeast and have concluded that this substance possesses biotin activity per se. A series of biotin analogues has been synthesized and activity tested, both as sources of biotin and as biotin inhibitors (157).

A number of workers have reported the substitution of fatty acids and lipid substances for biotin (158, 159, 160). In addition,

oleic acid has been reported as a growth factor for certain lactic acid bacteria (161, 162). The relationship of the fatty acid substitution to biotin function has not as yet been clarified.

Pantothenic acid.—Novelli & Lipmann (163) have given the title "coenzyme A" to a functional form of pantothenic acid, which serves as the coenzyme for acetylation. A test system for acetylation of aromatic amines by liver preparations has been adapted to the assay of the coenzyme by Lipmann (164). During coenzyme A purification, only traces of B vitamins were found; however, combined treatment with phosphatase and a liver enzyme was shown to liberate pantothenic acid (165).

Based on the observation made as early as 1942 by Dorfman *et al.* (166), namely, that pantothenic acid influenced the rate of pyruvate oxidation by *Proteus morgani*, Novelli & Lipmann (163, 167) incubated deficient cells of *Proteus morgani* with pantothenic acid and observed increased levels of coenzyme A in the cells. A parallel increase in pyruvate oxidation was found. *Lactobacillus arabinosus* was also found to convert pantothenic acid to coenzyme A, over 90 per cent of the pantothenic acid being present in this form. In a survey of natural materials as sources of coenzyme A, Lipmann *et al.* (168) found a general distribution, with the clostridia ranking among the richest sources. It now appears, from the studies of Lipmann *et al.* (169), that coenzyme A functions as the general coenzyme for acetylation, and since acetate is known to occupy an important place in the formation and degradation of fatty acids, and in oxidative pathways for carbohydrates, this coenzyme assumes even greater importance.

Ravel & Shive (170) reported the prevention of pantothenic acid synthesis by cysteic acid, a competitive inhibitor of aspartic acid metabolism. The mechanism suggested is the inhibition of β-alanine formation, which may serve as a precursor of pantothenic acid. β-alanine completely relieves the inhibition by cysteic acid, as does glutamic acid.

Shive *et al.* (171) have also reported a biosynthesis requiring pantothenic acid. The inhibition of *Escherichia coli* growth in the presence of cysteic acid can be reversed by citric, *cis*aconitic, and α-keto glutaric acids. Oxaloacetic and pyruvic acids are inactive, but the two together give slight activity, as does acetate. From these studies, it is concluded that one of the functions of pantothenic acid is in the condensation of acetate or similar compound

in the tricarboxylic acid system. For *Lactobacillus arabinosus*, oleic acid, in the form of Tween 80, increases the antibacterial index in the presence of cysteic acid, thus suggesting a function of pantothenic acid in acetate condensation to form fatty acids. The interpretation of pantothenic acid deficiency as an interruption in oxidative metabolism at the condensation step is in line with the acetylation studies of Lipmann and co-workers (163, 169).

Vitamin B_6 group.—In extending the observations of the function of Vitamin B_6 in the formation of tryptophane, Schweigert (172) has studied the use of indole and anthranilic acid for the growth of *Lactobacillus arabinosus*. Pyridoxal and pyridoxamine are essential for the synthesis of tryptophane from either substance; furthermore, serine and acetate were shown to increase the rate of tryptophane synthesis. Thus growth studies with *Lactobacillus arabinosus* has extended the studies with cell-free preparations of *Neurospora*, which indicated the function of pyridoxal phosphate in tryptophane synthesis (173). A further function of pyridoxal phosphate as the coenzyme of the tryptophanase system of *Escherichia coli* has been reported by Wood, Gunsalus & Umbreit (134), as discussed more fully under amino acid metabolism. Dawes, Dawson & Happold (131) have also observed the function of pyridoxal phosphate in the tryptophanase system although their work does not indicate quite so simple a system as that expressed above.

Lyman *et al.* (174) have extended the observations on the substitution of the Vitamin B_6 group for certain amino acids in the growth of lactic acid bacteria and have shown that carbon dioxide is also an important factor.

p-Aminobenzoic acid.—Shive and co-workers (175) by the use of partial sulfanilamide inhibition were able to isolate a purine precursor. Upon relief of the inhibition by *p*-aminobenzoic acid, the precursor failed to accumulate. The complete synthesis of purines would require ring closure by the addition of one carbon, thus indicating a possible function of PAB in carbon transfer.

Unidentified factors.—Following the studies of Miller *et al.* (176) on the pyruvic acid metabolism of streptococci, O'Kane & Gunsalus (177) have found that *Streptococcus faecalis*, strain 10 Cl, harvested from a synthetic medium adequate for growth, exhibited a depressed rate of pyruvate oxidation unless a factor present in yeast extract was added. Stimulations of tenfold or more were not uncommon. Partial fractionation of yeast extract revealed that the

active factor was adsorbed by charcoal and eluted with alkaline agents. It was soluble in a number of organic solvents and precipitated by certain heavy metals. The extreme stability of this substance to acid, alkali, and heat differentiates it from known factors, none of which has been found to replace it for pyruvate oxidation. Since coenzyme A was first known to function in pyruvate oxidation, it was tested but was not found to be active.

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THE METABOLISM OF MALARIAL PARASITES[1]

By James W. Moulder

Department of Bacteriology and Parasitology and Department of Biochemistry
The University of Chicago, Chicago, Illinois

Studies on the metabolism of malarial parasites are of interest not only because of their application to the rational chemotherapy of malaria but also because of their contribution to the understanding of the biochemical basis of obligate intracellular parasitism. The first direct investigation of malarial parasite metabolism was not reported until 1938 (1), but the military importance of malaria in the recent war greatly stimulated research on the metabolism of the parasites and on the metabolic effect of antimalarial drugs. Certain aspects of this wartime research have been summarized (2 to 5), but the general subject of malarial parasite metabolism has not been reviewed.

Methods of Study

The malarial parasites are all included within the single genus *Plasmodium*. These protozoan parasites undergo a cycle of asexual reproduction and produce gametocytes within the erythrocytes of vertebrates. The gametocytes, or sexual stages, develop no further in the vertebrate host, and fertilization occurs only in the invertebrate mosquito host. After fertilization, sporozoites are developed which are inoculated by the mosquito into a new vertebrate host. The sporozoites either directly, or more probably after passing through several exoerythrocytic stages, infect the erythrocytes to complete the life cycle. The classical studies on the life cycle of malarial parasites in the mosquito and in vertebrate erythrocytes have been fully described by Wenyon (6), and Huff (7) has discussed the exoerythrocytic development of plasmodia in the vertebrate host. Since the number of parasites obtainable in mosquitoes and in the exoerythrocytic stages in vertebrates is insufficient for metabolic study, only the blood stages in the life cycle of malarial parasites have been subjected to metabolic investigation. Blood stages include both asexual forms and gametocytes, but in the early period of blood-induced infections—the type of infection universally used as the source of parasites for biochemical study—

[1] This review covers the literature through December, 1947.

few gametocytes are present. Malarial parasites have been found in reptiles, birds, monkeys, and man, but only investigations on the metabolism of avian and simian parasites have been published.

Survival and growth of malarial parasites.—Although Bass & Johns reported the successful artificial cultivation of the erythrocytic forms of human malarial parasites in 1912, subsequent attempts to grow plasmodia *in vitro* were generally unsuccessful. These early experiments have been reviewed by Trager (8).

Trager (8, 9) devised conditions suitable for the survival and development of *P. lophurae in vitro.* Parasitized chick erythrocytes were suspended in a balanced salt solution containing erythrocyte extract glucose, glutathione, calcium pantothenate, serum, and embryo extract and incubated in air at 40°C. Each component of the medium exerted a favorable effect on survival of the parasite. Survival of the parasites was judged by their infectivity when inoculated into chicks and by exflagellation of the male gametocytes. The parasites continued to develop for about a week and some survived as long as sixteen days. Only very rarely did the total parasite number increase during cultivation. Coulston (10) has briefly described the cultivation of *P. circumflexum* by a method similar to Trager's.

Geiman *et al.* (11, 12) consistently obtained *in vitro* growth and multiplication of *P. knowlesi* by two different cultivation techniques. Their rocker-dilution technique is similar to that of Trager (8) and is well adapted to routine investigations. In the second more complicated procedure, the rocker-perfusion technique, a cellophane membrane separates whole parasitized blood from the nutrient medium. This technique is particularly useful in studies on the nutritional requirements for dialyzable substances. The usual medium consisted of a balanced salt solution containing serum, glucose, *p*-aminobenzoic acid, amino acids, vitamins, purines, pyrimidines, and other organic compounds. The parasites failed to multiply in the absence of either glucose or *p*-aminobenzoic acid, and omission of whole groups of other nutrients diminished parasite growth. In the first twenty-four hour asexual cycle, *P. knowlesi* showed an average fourfold increase in number, and the appearance of the parasites was normal. When the parasites were subcultured into fresh media every day, multiplication continued for five to six generations and the parasites in the final subculture were still highly infective. Trager (13) has recently

obtained active multiplication of *P. lophurae in vitro* by a procedure resembling both his original method (8, 9) and that of Geiman *et al.* (11, 12).

Hawking (14) made tissue cultures from the spleen, bone marrow, buffy coat, and other organs of chickens with sporozoite-induced infections of *P. gallinaceum* and observed active multiplication of exoerythrocytic stages for as long as eighty-nine days.

Short-term studies of parasite metabolism.—Many aspects of parasite metabolism are best investigated in experiments of one to six hours duration in systems with an approximately constant parasite population. Several different types of parasite preparations have been used in these metabolic studies:

(*a*) Whole blood (1, 15 to 18).—Whole parasitized blood containing a suitable anticoagulant may be used directly. Such a procedure is convenient and causes a minimum of damage to the parasites. Most plasmodia continue to respire at an undiminished rate for several hours after the parasitized blood has been drawn. The use of whole blood preparations for metabolic investigations has two disadvantages: the metabolism of a single added substrate cannot be studied, and the buffering capacity of the serum complicates the manometric measurements of gas exchange.

(*b*) Washed parasitized erythrocytes (1, 15 to 22).—Parasitized erythrocytes may be separated from whole blood by centrifugation and washed several times in buffered saline without significant reduction in metabolic activity. In the presence of a suitable subsubstrate, washed parasitized erythrocytes consume oxygen at a constant rate for several hours and apparently carry out all the metabolic reactions occurring in whole blood. Removal of the serum from parasitized erythrocytes reduces the retention of carbon dioxide in manometric measurements and allows the study of the response to any desired substrate. When the number of parasitized erythrocytes in a blood sample is too low for convenient study, the parasitized erythrocytes may be partially separated from unparasitized erythrocytes by fractional centrifugation (1, 18, 23).

(*c*) Free parasites.—Preparations of whole parasitized blood and washed parasitized erythrocytes contain both the malarial parasite and the host erythrocyte and are ideally suited to study of the general course of parasite metabolism and the biochemical nature of the host-parasite relationship in malaria. However, sev-

eral investigators have separated the parasites from the erythrocytes in order to study the metabolism of the plasmodia themselves. Christophers & Fulton (24) and McKee *et al.* (18) freed *P. knowlesi* from monkey erythrocytes by saponin hemolysis, and Bovarnick, Lindsay & Hellerman (25) also used saponin to liberate *P. lophurae* from duck erythrocytes. Speck, Moulder & Evans (22) hemolyzed chicken erythrocytes parasitized with *P. gallinaceum* with antichicken erythrocyte hemolysin from rabbits in order to obtain free parasites. Parasites prepared by these methods appeared normal and were free from erythrocyte stromata. However, such free parasite preparations were much less infective when subinoculated than were comparable preparations of parasitized erythrocytes. Certain polar substrates and cofactors without effect upon parasitized erythrocytes increase the rate of oxygen consumption of free parasites. These differences may reflect the relative impermeability of the erythrocyte membrane to such polar substances (26). The great disadvantage to the use of free parasites lies in their lessened and rapidly declining rate of oxygen uptake.

(*d*) Cell-free extracts (20, 27).—Finally, free parasites have been disrupted by grinding in a bacterial mill and their soluble proteins extracted with buffered saline. Both glycolytic and proteolytic enzymes have been found in such cell-free extracts. Glycolytic enzymes have also been demonstrated in preparations of parasitized erythrocytes lyzed with water (27).

The metabolic activity of parasitized blood or washed parasitized erythrocytes represents the sum of the activities of the parasites themselves and of the parasitized and unparasitized erythrocytes. In primate malarias, the metabolism of normal mammalian erythrocytes is negligible, but in avian malarias, the nucleated avian erythrocytes contribute significantly to the activity of preparations of parasitized erythrocytes. In all malarias, parasitized blood must be obtained from the host early in the infection before the appearance of reticulocytes in the circulating blood, because both mammalian and avian reticulocytes consume oxygen five to forty times as fast as normal erythrocytes (17, 21, 28, 29, 30). In the early stages of infection, the number of leucocytes present in the blood is too small to interfere with studies on parasite metabolism. Even when the erythrocyte is disrupted, as

in free parasite preparations or cell-free extracts, normal erythrocyte enzymes may still be present (22, 27, 31).

Oxygen uptake, the most widely measured metabolic activity, has been reported in terms of a unit volume of parasitized erythrocytes or free parasites, of the total number of erythrocytes, and of the total number of parasites. Of these, the expression of oxygen uptake in terms of parasite number is probably the most satisfactory. However, the magnitude of respiration of a constant number of plasmodia is dependent on their size. The development of a uninucleated merozoite into a multinucleated segmenter involves a manyfold increase in the size of the parasite and several investigators (15, 16, 17, 21) have noted that the respiration of the large forms is much greater than that of the small parasites. Silverman *et al.* (17) believe the metabolic activity of malarial parasites is best expressed in terms of the area of parasites in dried smears, which may be calculated from the total parasite number and the differential count. Their conclusion is supported by the work of Velick (21), who showed that the increase in respiration of *P. cathemerium* during a single asexual cycle closely parallels the increase in area of the parasites. When the effect of the number and size of parasites in a given preparation is considered, the respiration of all species of *Plasmodium* so far studied is roughly the same and the results obtained for a single species by different investigators are in reasonable agreement. Most of the reported values fall within the range of 150 to 400 μl. of oxygen per 10^9 parasites per hour.

Metabolism of Malarial Parasites

Oxygen transport.—Much of our knowledge of the nature of oxygen-transporting enzymes in plasmodia has been gained from studies with respiratory inhibitors. Christophers & Fulton (1) reported that the oxygen consumption of *P. knowlesi* is almost completely inhibited by 0.001 *M* cyanide and their report has been confirmed by Wendel (16) and McKee *et al.* (18). Bovarnick, Lindsay & Hellerman (25) found that the respiration of *P. lophurae* also is almost completely cyanide-sensitive. McKee *et al.* (18) observed that the oxygen uptake of monkey erythrocytes parasitized with *P. knowlesi* in 95 per cent carbon monoxide—5 per cent oxygen is only 36 per cent of the uptake in 95 per cent nitrogen—5

per cent oxygen. They were unable to obtain consistent reversal of the carbon monoxide inhibition upon irradiation, possibly because of the great light-absorbing capacity of the parasitized erythrocyte suspension. Respiration of *P. lophurae* is less inhibited by azide than by cyanide (25). Velick (21) found that parasitized erythrocytes (*P. cathemerium*) oxidize *p*-phenylenediamine faster than normal erythrocytes. These observations suggest that malarial parasites contain iron-porphyrin respiratory enzymes of the Warburg-Keilin system, but the possible presence of copper-protein respiratory enzymes has not been ruled out.

To demonstrate flavoprotein enzymes in *P. lophurae*, Bovarnick *et al.* (25) studied the effect of cresyl blue and similar dyes on oxygen uptake in the presence of cyanide. They found that cresyl blue restores the cyanide-sensitive respiration to about 40 per cent of the original rate. Failure to restore completely the normal respiratory rate with cresyl blue was believed due to the inhibitory action of the dye upon the normal respiration of the parasites. Ball *et al.* (32) observed increases in the flavin adenine dinucleotide content of monkey erythrocytes parasitized with *P. knowlesi* during growth of the parasites both *in vivo* and *in vitro*.

Lactic (27) and malic (22) dehydrogenases requiring diphosphopyridine nucleotide for maximum activity have been found in *P. gallinaceum*, and both di- and triphosphopyridine nucleotides increase the rate of pyruvate oxidation in free parasite preparations of this organism (22).

In general, all these studies support the tentative conclusion that oxygen transport in malarial parasites is catalyzed by iron-porphyrin proteins, flavoproteins, and pyridinoproteins similar to those demonstrated in other organisms. High oxygen tensions inhibit respiration (17, 18) and *in vitro* growth (8, 12) of plasmodia, but the mechanism of inhibition is unknown.

The role of carbon dioxide in the metabolism of malarial parasites.—Plasmodia have been cultivated in bicarbonate media, but most short-term studies of parasite metabolism have been carried out in carbon dioxide-free phosphate media. Although carbon dioxide is known to participate in many enzymic reactions in other organisms, no differences have been observed in the metabolism of malarial parasites in the presence or absence of carbon dioxide.

Respiration of malarial parasites in the absence of identifiable substrates.—Christophers & Fulton (1) found that washed parasit-

ized erythrocytes (*P. knowlesi*) continue to consume oxygen at a constant rate for several hours in the absence of detectable amounts of glucose or other reducing substances. The R.Q. of the respiration is 0.9. Also working with *P. knowlesi*, Maier & Coggeshall (15) and McKee *et al.* (18) observed that the oxygen consumption of parasitized erythrocytes in the absence of glucose is about half of the uptake obtained with glucose. The respiration of free parasites of *P. lophurae* in the absence of glucose is relatively small, amounting to only about 10 per cent of the rate in its presence, and is increased upon addition of adenosinetriphosphate (25).

Speck, Moulder & Evans (22) studied the endogenous respiration of free parasites and parasitized erythrocytes from *P. gallinaceum* infections. In parasitized erythrocytes, the endogenous oxygen uptake is 40 to 50 per cent of the uptake on glucose, and the comparable value for free parasites is 25 to 50 per cent. In these preparations, glucose, pyruvate, malate, and citrate were completely absent. Lactate and total reducing substances after acid hydrolysis were present in insignificant amounts and did not change in concentration during the period of observation. The endogenous respiration of *P. gallinaceum* is strongly inhibited by malonate, and small amounts of succinate accumulate in the presence of malonate. These findings, together with the observed R. Q. of 0.9, suggest that the unknown substrate is carbohydrate in nature. It has been generally assumed that addition of rapidly oxidized substrates such as glucose, pyruvate, or lactate results in displacement of the endogenous respiration.

Carbohydrate metabolism of malarial parasites.—The importance of carbohydrates in the metabolism of malarial parasites is well illustrated by the observation that glucose, or other utilizable carbohydrate, is absolutely essential for the *in vitro* survival and growth of the parasites (8, 9, 11, 12, 13). The obvious importance of carbohydrate in the economy of plasmodia, together with our relatively advanced knowledge of the course of carbohydrate breakdown in other organisms, has resulted in intensive study of the carbohydrate metabolism of malarial parasites.

Although Christophers & Fulton (1) first reported that glucose does not increase the oxygen consumption of *P. knowlesi*, later work by Christophers & Fulton (24), as well as by Fulton (19), Maier & Coggeshall (15), Wendel (16), and McKee *et al.* (18),

proved that glucose greatly increases the respiration of washed preparations of *P. knowlesi*. All these investigators observed that glucose rapidly disappears from blood infected with *P. knowlesi*. Fructose, mannose, lactate, and glycerol are oxidized by *P. knowlesi* at rates comparable to the rate of glucose oxidation (15, 16, 18, 19, 33). Bovarnick *et al.* (25) found that free parasites of *P. lophurae* oxidize glucose, lactate, and pyruvate at approximately equal rates and attack succinate and fumarate at distinctly slower rates. Parasitized erythrocytes and free parasites of *P. gallinaceum* oxidize glucose, lactate, and pyruvate at equal rates (17, 22), and free parasites also rapidly oxidize succinate, fumarate, malate, oxaloacetate, *cis*-aconitate, and α-ketoglutarate (22). Glycogen does not increase the respiration of malarial parasites, but it is destroyed *in vitro* by *P. knowlesi* (1) and may replace glucose in the cultivation of *P. lophurae* (8).

The products of glucose breakdown by malarial parasites have been identified in *P. knowlesi* and *P. gallinaceum*. Wendel & Kimball (33) and Wendel (16) demonstrated that monkey blood infected with *P. knowlesi* rapidly converts glucose to lactate and pyruvate which are later partially removed by oxidation. At their maximum concentrations, the ratio between lactate and pyruvate was about three to one, and Wendel & Kimball suggested that high concentrations of pyruvate in infected blood are actually toxic to the parasites. McKee *et al.* (18) found that *P. knowlesi* first converts glucose into lactate and pyruvate and that these substances are then removed by oxidation at about one sixth the rate of their formation from glucose. From the observed oxygen/lactate ratio, they concluded that lactate is oxidized incompletely. Lactate does not disappear anaerobically. The accumulation of pyruvate observed by McKee *et al.* was much less than that found by Wendel & Kimball. In *P. gallinaceum* infections, Silverman *et al.* (17) snowed that parasitized erythrocytes break down glucose to lactate anaerobically and oxidize a portion of the lactate formed from glucose to carbon dioxide and water in air. About 85 per cent of the glucose utilized in air can be accounted for by the lactate formed and the carbon dioxide produced. The most striking feature of glucose breakdown in these two species is the rapid formation and accumulation of lactate, even under aerobic conditions.

Since the mechanism of glucose oxidation has been worked out

in greatest detail for *P. gallinaceum*, the results with this species will be described first. The observation of Silverman *et al.* (17) that glucose is quantitatively converted into lactate by parasitized erythrocytes under anaerobic conditions suggested that glucose is utilized in *P. gallinaceum* by means of a typical phosphorylating glycolysis. In confirmation of this suggestion, Speck & Evans (27) prepared cell-free extracts from *P. gallinaceum* which catalyzed the phosphorylation of glucose by adenosinetriphosphate, the cleavage of fructose-1,6-diphosphate to form 3-phosphoglyceraldehyde, and the coupled oxidation-reduction reaction between 3-phosphoglyceraldehyde and pyruvate.

Silverman *et al.* (17) showed that the lactate formed from glucose by *P. gallinaceum* is almost completely oxidized to carbon dioxide and water under aerobic conditions and that glucose, lactate, and pyruvate are all oxidized at the same rate. Thus, in its carbohydrate metabolism, *P. gallinaceum* resembles other organisms in which glucose oxidation is known to proceed through pyruvate as an intermediate. The most generally distributed mechanism for pyruvate oxidation in other organisms is the tricarboxylic acid cycle of Krebs. Since chicken erythrocytes may be impermeable to the highly polar acids of the tricarboyxlic acid cycle, Speck, Moulder & Evans (22) studied the pyruvate metabolism of free parasites as well as of parasitized erythrocytes of *P. gallinaceum*. In both preparations pyruvate is oxidized to carbon dioxide and water by a cycle like that proposed by Krebs for muscle tissue. In free parasites, the acids of the tricarboxylic acid cycle, succinate, fumarate, malate, oxaloacetate, *cis*-aconitate, and α-ketoglutarate are oxidized at rates comparable to the rate of pyruvate oxidation. The oxidation of pyruvate is catalyzed by the dicarboxylic acids and is strongly inhibited by malonate with the accumulation of succinate. The malonate inhibition may be removed by the addition of fumarate. In parasitized erythrocytes, the oxidation of pyruvate is inhibited by malonate, and succinate is formed. In both types of parasite preparations, pyruvate does not disappear anaerobically, and the oxidation of glucose and lactate is also inhibited by malonate. In addition to complete oxidation via a tricarboxylic acid cycle, glucose, lactate, and pyruvate are also oxidized to acetate by *P. gallinaceum*, but it is unlikely that appreciable amounts of acetate are formed by the parasites under physiological conditions.

These investigations indicate that, in *P. gallinaceum*, glucose is converted to pyruvate by a phosphorylating glycolysis; the pyruvate is oxidized to carbon dioxide and water through a tricarboxylic acid cycle or is reversibly reduced to lactate. The mechanism of carbohydrate oxidation in *P. knowlesi* and *P. lophurae* is not known in comparable detail, but some pertinent observations concerning the carbohydrate metabolism of these species may be compared with the results obtained with *P. gallinaceum*. Although Christophers & Fulton (1) reported that lactate is not utilized by *P. knowlesi* and Maier & Coggeshall (15) stated that lactate produces only about half the increase in oxygen consumption produced by glucose, both Wendel (16) and McKee *et al.* (18) found that lactate is as good a substrate for *P. knowlesi* as is glucose. Lactate and pyruvate are formed from the breakdown of glucose in *P. knowlesi* (16, 18). Wendel (16) failed to obtain inhibition of glucose oxidation in blood infected with *P. knowlesi* by the addition of malonate. According to Bovarnick *et al.* (25) free parasites of *P. lophurae* oxidize glucose, lactate, and pyruvate at equal rates. Succinate and fumarate are oxidized more slowly. In substrate-depleted preparations of *P. lophurae*, adenosinetriphosphate, adenylic acid, succinate, and fumarate increase the rate of glucose oxidation (34). Hellerman, Bovarnick & Porter (4) found that malonate inhibits glucose oxidation in free parasites of *P. lophurae* by 30 per cent, pyruvate oxidation by 50 per cent. Therefore, despite the lack of detailed formation and the presence of a few contrary observations, it appears likely that the mechanism of glucose oxidation in *P. knowlesi* and *P. lophurae* is similar to that demonstrated in *P. gallinaceum*.

Although it is generally assumed that in aerobic organisms such as the malarial parasites most of the energy available for growth and development comes from the aerobic oxidation of carbohydrate, Ball and his associates have suggested that the energy released in the anaerobic breakdown of glucose may also be used by malarial parasites in meeting the energy requirements for growth. Ball *et al.* (3, 32) found that the *in vitro* growth of *P. knowlesi* is accompanied by a much smaller increase in oxygen uptake than is observed during a comparable period of growth *in vivo*, and at an oxygen concentration of 0.37 per cent, Anfinsen *et al.* (12) obtained *in vitro* growth of *P. knowlesi* which was as good as that obtained with oxygen tensions of 5 per cent and higher.

Protein metabolism of malarial parasites.—Since malarial parasites rapidly increase in size and number in the blood of the host, they must synthesize large amounts of protein. Studies on the protein metabolism of plasmodia indicate that the nitrogen used in the synthesis of parasite protein is obtained from the hemoglobin of the erythrocyte and from the serum.

The relation of pigmented bodies in the spleen, liver, and blood to malaria was recognized by the early pathologists long before Laveran's discovery of the malarial parasite. After the nature of malaria was known, it slowly became evident that the pigment present in parasites and in infected tissues is formed by the action of the malarial parasite upon the hemoglobin of the erythrocyte [for a review of the early literature, see (35)]. Brown (36) offered strong evidence that the pigment in tissues from human malaria cases is identical with hematin. In a series of studies on the chemical and physical nature of the pigment isolated from monkey erythrocytes parasitized with *P. knowlesi*, Sinton & Ghosh (35, 37, 38) and Ghosh & Nath (39) conclusively demonstrated that this substance is hematin. Other workers have also identified the pigment of *P. knowlesi* (40, 41) and of *P. gallinaceum* (42) as hematin.

Brown (36) suggested in 1911 that malarial parasites contain an enzyme capable of splitting hemoglobin into globin and hematin, the globin being further metabolized in the economy of the parasites and the hematin being merely a waste product. Christophers & Fulton (1) showed that the oxyhemoglobin of monkey erythrocytes parasitized with *P. knowlesi* disappears during incubation *in vitro*, while the oxyhemoglobin of unparasitized erythrocytes does not. By use of a histological method for detection of hemoglobin, Black (43) found that several different species of *Plasmodium* destroy hemoglobin. To learn the fate of the globin presumably liberated when hemoglobin is split into globin and hematin, Christophers & Fulton (1) measured the appearance of amino nitrogen, ammonia, and nonprotein nitrogen in parasitized erythrocytes during incubation. They observed significant increases in the nonprotein nitrogen content of parasitized erythrocytes, small changes in amino nitrogen, and no changes in ammonia. Working with *P. gallinaceum* in the chicken, Moulder & Evans (20) found that, unlike normal erythrocytes, parasitized erythrocytes produce large amounts of amino nitrogen aerobically

in the presence of glucose. When glucose is absent from the medium much of the amino nitrogen appears as ammonia. The formation of amino nitrogen in parasitized erythrocytes is greatly decreased under anaerobic conditions, but cell-free extracts of *P. gallinaceum* contain enzymes which hydrolyze globin at the same rate in the presence or absence of air. Therefore, Moulder & Evans suggested that hydrolysis of protein is in some manner linked to oxidative processes in the intact malarial parasite. The proteinases present in extracts of *P. gallinaceum* hydrolyze denatured globin much faster than native hemoglobin at pH 7, and their activity is not increased by reducing agents such as cyanide. These observations on *P. knowlesi* and *P. gallinaceum* support Brown's view that the malarial parasite enzymatically splits hemoglobin into hematin and globin and suggest that the globin so formed is further hydrolyzed to amino acids which may be used in the synthesis of parasite protein or deaminated with liberation of ammonia.

Studies on the growth of plasmodia outside of the vertebrate host should answer many questions concerning the protein metabolism of the parasites, since the metabolism of protein is intimately associated with growth. Ball and his co-workers (3, 12, 32) have already shown by their cultivation technique that the presence of amino acids, purines, and pyrimidines in the medium are necessary for optimal growth of *P. knowlesi in vitro*. As a step toward simplification of their medium for further nutritional studies, they have succeeded in cultivating *P. knowlesi* in monkey erythrocytes washed free of serum and suspended in 1 per cent crystalline egg albumin (12).

Other metabolic investigations.—The rapid growth of plasmodia within erythrocytes is accompanied by the formation of large amounts of nuclear material as demonstrated by standard staining methods. Ball *et al.* (3, 32) more directly showed that the nucleic acid content of parasitized erythrocytes greatly increased during *in vivo* or *in vitro* multiplication of the parasites by measuring the changes in the nucleic acid phosphorus content of monkey erythrocytes infected with *P. knowlesi*. Miller & Kozloff (31) reported that the washed residues from normal chicken erythrocytes hemolyzed with saponin slowly depolymerize ribonucleic acid, while free parasites of *P. gallinaceum* depolymerize ribonucleic acid at a faster rate, and, in addition, further degrade ribonucleic acid to nucleotides and inorganic phosphorus. By use of the Feulgen

technique, Deane (44) showed that part of the nuclear material in *P. vivax* and *P. knowlesi* is desoxyribonucleic acid.

Significant increases in nucleic acid phosphorus, phospholipid phosphorus, and fifteen-minute acid hydrolyzable phosphorus occur during the growth of *P. knowlesi* within monkey erythrocytes (3, 32). When free parasites of *P. lophurae* are incubated in the absence of added substrate, there is an increase in the inorganic and acid-soluble phosphorus levels and a decrease in the organic and labile phosphorus levels (34). When glucose is added, these changes are partially reversed.

Ceithaml & Evans (45) exposed parasitized erythrocytes (*P. gallinaceum*) to 30,000 r units of x-ray and observed an increase in the rate of oxygen consumption and glucose utilization.

Some general aspects of parasite metabolism.—All the metabolic activities of the blood stages of malarial parasites so far described may be divided into three main groups: (*a*) glycolysis of glucose to lactate and complete oxidation of much of the lactate to carbon dioxide and water via pyruvate as an intermediate; (*b*) cleavage of hemoglobin to hematin and globin, hydrolysis of globin to amino acids, and utilization of the free amino acids; and (*c*) synthesis of protein, nucleic acid, lipid, and other cell substances. This pattern of metabolism might logically be expected of a rapidly multiplying organism; that is, the metabolism of malarial parasites appears primarily directed toward production of the energy and the cellular material essential for growth.

Malarial parasites are obligate intracellular parasites, for the blood stages of plasmodia grow and multiply only within the erythrocytes of their specific hosts. Obligate intracellular parasitism represents an extreme form of parasitic adaptation, and adaptation to a parasitic existence is usually thought of as occurring through the progressive loss of enzymes no longer essential for survival in the new environment. Yet it is impossible to point out where parasitic adaptation and enzyme loss has occurred in *Plasmodium*. Almost every type of enzymic reaction discovered in higher animals has been found in malarial parasites, and the known growth factors required by plasmodia are similar to those needed by many extracellular parasites and saprophytes. Therefore, we cannot at present explain why the rapidly metabolizing blood stages of malarial parasites live and grow only within erythrocytes, which have much lower metabolic rates.

Trypanosomes are the only other group of parasitic protozoa which have been subjected to intensive metabolic study. Comparison of the intracellular malarial parasites with the extracellular trypanosomes suggests that trypanosomes have lost more enzyme systems through parasitic adaptation than have the plasmodia. Trypanosomes oxidize glucose very incompletely, and the pathogenic species produce almost no carbon dioxide from glucose (1, 46); they have no oxidizable reserve substances and do not respire in the absence of added substrates (1, 46, 47); they do not hydrolyze proteins under physiological conditions (47, 48); and the pathogenic trypanosomes do not have cyanide-sensitive respiratory enzymes (49).

The metabolism of malarial parasites greatly resembles that of their vertebrate hosts. Oxygen is transported by enzymes similar to the familiar respiratory enzymes of higher animals. The utilization of glucose in parasites and in vertebrate muscle proceeds along almost identical pathways. Vitamins, cofactors, etc., active in the vertebrate host are also involved in the metabolism of the parasite. In short, with the exception of the cleavage of hemoglobin to hematin and globin, no enzymic reaction has been found in malarial parasites which does not have its counterpart in higher animals. These metabolic similarities must be considered in attempting to inhibit selectively the metabolism of malarial parasites with chemotherapeutic agents.

The Effect of Antimalarial Drugs on the Metabolism of Malarial Parasites

The literature on antimalarial drugs was reviewed last year by Blanchard (50) and by Lourie (51). The results of the assays of substances with potential antimalarial activity which were conducted under the auspices of the Committee on Medical Research of the Office of Scientific Research and Development have now been summarized in a monograph entitled *A Survey of Antimalarial Drugs* (52). Because of these recent general articles on the chemotherapy of malaria, the following discussion will be limited to consideration of the effects of antimalarial drugs on the metabolism of malarial parasites.

Of the many chemotherapeutic agents active against malaria, the action of quinine, quinacrine (atebrin), the sulfonamides, and the 2-hydroxy-3-alkylnaphthoquinones on the metabolism of

plasmodia has been studied in sufficient detail to warrant discussion.

Quinine.—In concentrations of 0.001 to 0.0001 *M*, quinine inhibits the oxygen consumption of avian and simian malarial parasites by 25 to 50 per cent (17, 53, 54). Silverman *et al.* (17) found that both the aerobic and anaerobic formation of lactate from glucose by *P. gallinaceum* is inhibited by 0.001 *M* quinine. In their study of the glycolytic enzymes present in extracts from *P. gallinaceum*, Speck & Evans (55) observed that the enzymes, hexokinase and lactic dehydrogenase, are inhibited by 0.001 *M* quinine. It is probable, that at this concentration, quinine inhibits glycolysis in *P. gallinaceum* by its action on these enzymes.

However, the quinine concentration necessary to inhibit glycolysis is one hundred to one thousand times greater than the level in the serum of experimental animals during quinine therapy (56, 57). Ceithaml & Evans (58) found that the quinine concentration in parasitized erythrocytes is much higher than in serum, but the observed intracellular/extracellular quinine ratio was never greater than fifty. At 0.00001 *M*, about the serum concentration in quinine therapy, quinine inhibits the oxygen consumption of chicken erythrocytes parasitized with *P. gallinaceum* but does not inhibit the formation of lactate from glucose (17). On the basis of this observation, it was suggested that quinine inhibits some step in the oxidation of pyruvate to carbon dioxide and water.

Quinacrine.—The addition of 0.001 to 0.0001 *M* quinacrine reduces the oxygen uptake of malarial parasites by about one half (17, 53, 54). As with quinine, these concentrations are roughly one thousand times higher than the serum levels obtained in the treatment of experimental malaria infections with quinacrine (59). In high concentrations, quinacrine inhibits both respiration and glycolysis in *P. gallinaceum;* lower concentrations inhibit only respiration (17).

Wright & Sabine (60) found that quinacrine inhibits the flavoprotein enzyme D-amino acid oxidase and that the inhibition is antagonized by flavin adenine dinucleotide. Similarly, Haas (61) reported that the flavoprotein enzyme, cytochrome reductase, is strongly inhibited by quinacrine and that the inhibition may be prevented by riboflavin phosphate. These observations, together with the similarity in structure between quinacrine and the flavin nucleotide coenzymes, suggested that quinacrine might specifically

compete with the flavin coenzymes for the enzyme proteins. Hellerman, Lindsay & Bovarnick (62), upon investigation of this possibility, found that quinacrine inhibits D-amino acid oxidase strongly at low concentrations of flavin adenine dinucleotide and hardly at all at high concentrations of this coenzyme. However, they also found that quinacrine shares this property with many other compounds, some completely unrelated in structure to the flavin nucleus and without antimalarial activity. Of the compounds studied, quinacrine, quinine, plasmochin, and certain quinoline derivatives were the most effective as flavoprotein enzyme inhibitors. Hellerman *et al.* (62) concluded that quinacrine interferes with the activity of flavoproteins, not because of its structural resemblance to the flavin coenzymes, but because of its ability to combine with the enzyme proteins, an ability possessed by many other substances.

Speck & Evans (55) found that the phosphorylation of glucose by the enzyme hexokinase in extracts of *P. gallinaceum* is strongly inhibited by quinacrine. Bovarnick, Lindsay & Hellerman (34) have also concluded that quinacrine interferes with the phosphorylation of glucose by *P. lophurae.* The oxidation of glucose by free parasites of *P. lophurae* is only slightly inhibited by 0.0001 *M* quinacrine; but when the free parasites are depleted of substrates by maintenance at 38°C. for one hundred minutes in the absence of glucose, the oxidation of added glucose is inhibited 75 to 90 per cent by 0.0001 *M* quinacrine. Such substrate-depleted free parasites oxidize glucose only after an induction period which may be shortened by the addition of adenylic acid, adenosinetriphosphate, succinate, or fumarate. The quinacrine inhibition of oxygen consumption may be almost completely abolished by the simultaneous addition of adenosinetriphosphate or adenylic acid. Bovarnick *et al.* (34) have suggested that quinacrine interferes with the phosphorylation of glucose, possibly by competition with adenosinetriphosphate for the enzyme hexokinase.

Sulfonamides.—Several sulfonamides inhibit the oxygen consumption of malarial parasites *in vitro* (21, 54, 63). In *P. knowlesi* infections, Coggeshall & Maier (54) found that the sodium salts of sulfathiazole and sulfapyridine in 0.01 *M* concentrations inhibit the respiration of the parasites by about 40 per cent. Sulfanilamide is less effective as a respiratory inhibitor. Several observations suggest that the sulfonamide inhibition of respiration in

plasmodia is similar in mechanism to the inhibition of respiration and growth in bacteria and other organisms. Marshall, Litchfield & White (64) and Maier & Riley (65) have shown that the chemotherapeutic action of sulfonamides against malaria is antagonized by *p*-aminobenzoic acid. As already mentioned, Ball and his associates (12) obtained *in vitro* growth of *P. knowlesi* only in the presence of *p*-aminobenzoic acid. They further demonstrated that sulfadiazine inhibits the growth of *P. knowlesi* in artificial culture and that this inhibition may be prevented by the simultaneous addition of adequate amounts of *p*-aminobenzoic acid to the culture medium (3).

2–Hydroxy–3–alkylnaphthoquinones.—In studies not yet published in full, Wendel (5) discovered that many 2-hydroxy-3-alkylnaphthoquinones, which are active against infections with *P. lophurae* and *P. knowlesi*, strongly inhibit the respiration of these parasites at concentrations as low as 0.000001 *M*. Glucose utilization is not affected, but lactate oxidation is inhibited by effective naphthoquinones. Ball, Anfinsen & Cooper (66) confirmed Wendel's results with *P. knowlesi* and observed that the respiration of yeast cells and the activity of beef heart succinoxidase are also inhibited by low concentrations of naphthoquinones. Respiratory enzymes other than the succinoxidase system are not affected. Upon analysis of the effect of naphthoquinones on respiration, Ball *et al.* (66) concluded that these substances inhibit some step in the main chain of electron transport located below cytochrome-*c* and above cytochrome-*b*.

When investigations of the effect of antimalarial drugs upon parasite metabolism were initiated by Fulton & Christophers (53), it was hoped that the inhibition of metabolic processes by a substance might offer a convenient method for evaluating its therapeutic effectiveness, but it has been found that the effectiveness of drugs against various malarias often does not parallel the inhibition of parasite metabolism (5, 53, 54). However, fundamental studies on the metabolism and nutrition of plasmodia may answer some of the puzzling problems encountered in the chemotherapy of malaria, such as the differential action of drugs on exoerythrocytic and endoerythrocytic stages and the widely varying activity of a given drug against different species. Such work should also suggest new types of substances which may make effective antimalarial drugs. The discovery of the antimalarial activity of cer-

tain pantothenic acid analogues [see Lourie (51)], which was based on Trager's demonstration (9) of the beneficial effect of pantothenic acid on the survival of *P. lophurae in vitro*, may be cited as an example of what may be expected from continued study of the nutrition and metabolism of malarial parasites.

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GROWTH FACTORS FOR MICROORGANISMS

By Stewart A. Koser

Department of Bacteriology and Parasitology, University of Chicago
Chicago, Illinois

Comprehensive summaries of work in this field by Peterson & Peterson (1), Knight (2), and Snell (3) appeared in 1945 and 1946; consequently, this review will be limited to material that has appeared since these publications. The growth factor needs of the protozoa were reviewed recently by Lwoff (4) and will not be included here. The subject of inhibition of growth by compounds related chemically to essential metabolites also has been reviewed recently by several authors (5, 6) and will not be dealt with other than for purposes of occasional illustration of problems bearing upon the discussion.

Recent developments concerning growth factors for microorganisms have included studies of the needs of certain species or groups, the structure and functional role of some of the B vitamins, the activity of derivatives and analogues, variation in vitamin requirement, the synthesis of vitamins, bacterial destruction of vitamins, and the effect of massive doses of a vitamin.

Growth Factor Needs of Some Bacteria and Fungi

Bacillus and Clostridium.—A strain of *Bacillus anthracis* studied by Brewer and associates (7) requires thiamine for growth. The pyrimidine and thiazole components of thiamine are inactive. Uracil, adenine, and guanine are stimulatory and proper amounts of certain inorganic ions are important for securing heavy growth. Katznelson & Lochhead (8) found that *B. alvei* and *B. para-alvei*, bacteria associated with disease of bees, both need thiamine. The latter organism, however, grows moderately without thiamine in the presence of fourteen amino acids, of which phenylalanine, valine, isoleucine, and cystine are particularly important. The thiazole moiety of thiamine substitutes for these amino acids (9). This raises a question of interrelationship of thiamine and amino acids which has not as yet been clarified.

A strain of *Clostridium perfringens* produced luxuriant growth in casein digest or amino acid basal medium supplemented with a member of the vitamin B_6 group, pantothenate, thiamine, nico-

tinic acid, riboflavin, biotin, folic acid, adenine, guanine, uracil, salts, and glucose [Boyd, Logan & Tytell (10)]. Three strains of a saccharolytic type of *C. acetobutylicum* studied by Reyes-Teodoro & Mickelson (11) required biotin and one of them needed *p*-aminobenzoic acid as well. The biotin requirement of nine species of butyl alcohol-producing anerobes was studied by Perlman (12). Although biotin was required for maximum growth by all of the cultures, some of them produced appreciable growth in its absence. After ten to twenty daily transfers in a biotin deficient medium the biotin requirement was less marked. For *C. kluyveri*, the yeast autolysate formerly used may be replaced by biotin, *p*-aminobenzoic acid, and acetate [Bornstein & Barker (12a)]. In a simple medium containing these compounds together with ethanol and salts *C. kluyveri* grows very well under anaerobic conditions. The energy requirements of this "two-substrate" organism are satisfied by transformations of the ethanol and acetate. The pantothenate requirement of two strains of *C. septicum* was investigated by Ryan and co-workers (13). One strain required the preformed pantoyl moiety of the pantothenate molecule, the other was able to synthesize pantothenate. Several chemical analogues were studied as possible antivitamins. Three of them, sodium *dl*-pantoyltaurine, sodium γ-hydroxy pantoate, and pantoic amide possessed a considerable measure of growth promoting activity for the pantothenate deficient strain.

Brucella.—Heavier growth of an organism usually can be secured when the proper balance of vitamins, salts, and other components of the medium has been attained. Greatly increased cell yields of a strain of *Brucella suis* were secured in a chemically-defined medium by McCullough and associates (14) after an intensive study of the needs of this organism. In addition to thiamine, nicotinic acid, biotin, and pantothenate, previously recognized as essential or stimulatory for Brucella, the concentrations of inorganic ions, glucose, yeast nucleic acid or its components, and amino acids are important. For example, the use of appropriate amounts of thiamine, glucose, and iron salts in tryptose broth increased the cell yield five- to tenfold.

Cocci.—Progress has been made in the study of the nutritive needs of some of the more exacting cocci. The requirements of pneumococci were studied by Adams & Roe (15), who secured growth of most strains. The needs of individual strains differed,

but asparagine, glutamine, and choline were often necessary in addition to the other vitamins, purines, and pyrimidines used in previous studies. Among the gonococci some strains require for growth either cocarboxylase or thiamine monophosphate, due apparently to inability to phosphorylate thiamine [Lankford & Skaggs (16)]. These strains produced variants able to utilize thiamine. The inhibitory effect of agar on the gonococcus was shown by Ley & Mueller (17) to be due probably to its content of higher fatty acids. The beneficial effect of starch, recognized for many years in the cultivation of the gonococcus, is evidently an adsorption phenomenon. *Neisseria sicca* was shown by Ordal & Busch (18) to require biotin. *Streptococcus apis* requires pantothenic acid, nicotinic acid, pyridoxine, and biotin. One of the two strains studied also needs folic acid and is stimulated by thiamine [Katznelson(19)].

Lactic acid bacteria.—Although the lactic acid bacteria have received much attention in past studies they continue to be a lively topic of investigation. Pantothenic acid was required by thirty-three cultures studied by Cheldelin, Hoag & Sarett (20), the β-alanine and lactone moieties being insufficient. To maintain optimum growth of *Lactobacillus casei* during the first sixteen hours Scott and co-workers (21) report that strepogenin, glutathione, and a third unidentified factor present in casein hydrolyzate must be supplied, in addition to other compounds previously known to be needed. The study of lactic acid bacteria by Dunn, Shankman and associates (22, 23) is a valuable summary of the vitamin and amino acids needs of twenty-three different strains subjected to test at the same time and under uniform conditions. An oral *Lactobacillus* investigated by Weisberger & Johnson (24) grew well in casein hydrolyzate containing thiamine, pantothenate, and nicotinic acid. Several strains of *Microbacterium lacticum* required pantothenate for growth and thiamine was stimulatory though not essential (25).

The importance of oleic acid and some related compounds for lactobacilli has been stressed in recent papers (26 to 29). Some lactic acid bacteria require oleic acid, linoleic acid, or a combined source of these compounds in addition to other previously recognized factors; others require oleic acid or similar compounds only when biotin is omitted from the medium. Certain water-soluble emulsifying agents such as the Tweens[1] are more effective than

[1] The Tweens are fatty acid esters of polyoxyethylene derivatives of sorbitan.

oleic acid as growth promoting agents (28). In particular Tween 80, a water-soluble ester of oleic acid, is quite effective. The substitution of oleic acid and related compounds for biotin is discussed in the section on biotin.

The occurrence of other growth factors, at present unidentified, has been reported. Cheldelin & Riggs (30) called attention to a factor needed by *L. gayonii* and gave methods for its concentration. Hutchings, Sloane & Boggiano (31) studied the properties of this factor and consider it to be a stimulant rather than an essential factor. Barton-Wright, Emery & Robinson (32) described the separation, from by-products in the manufacture of commercial liver extract, of several fractions containing factors important in the growth of *L. helveticus* and *S. faecalis*. A "T" factor for *L. fermenti* was reported by Metcalf, Hucker & Carpenter (33). Two unidentified factors needed by *L. lactis* were noted by Shorb (33a). An unknown substance in some lots of peptone appeared necessary for growth of *L. plantarum* in the study of Camien, Dunn & Salle (34). While some of these unidentified factors eventually may be merged with one another or with known compounds, there is also the possibility that further study of them may lead to the recognition of important new compounds.

Phytopathogenic bacteria.—A study of the needs of an extensive series of phytopathogenic bacteria was made by Starr (35). Of thirty species and varieties of *Xanthomonas*, most were able to grow in an ammonium chloride, glucose, inorganic salts medium. For those strains unable to develop in this simple medium, nicotinic acid, glutamic acid, and methionine in various combinations supplied the needed preformed substances. *Agrobacterium radiobacter*, *A. tumefaciens*, and *A. gypsophilae* grew in the simple basal medium, but *A. rhizogenes* needed biotin and glutamic acid, and *A. rubi* needed the same two compounds and also nicotinic acid and pantothenate.

Photosynthetic bacteria.—Hutner (36) studied the organic growth factor needs of 124 strains of aerobic nonsulfur photosynthetic bacteria. Each of the five species of *Rhodospirillum* has a different requirement, but within each species the requirement is quite uniform for different isolates: *R. rubrum* needs biotin, *R. palustris* *p*-aminobenzoic acid, *R. capsulatus* thiamine, and, in certain media, biotin and nicotinic acid as well, *R. gelatinosa* thiamine and biotin, and *R. spheroides* thiamine, biotin, and nicotinic acid.

Molds.—For *Rhizopus suinus* Schopfer & Guilloud (37) report that thiamine accelerates growth and the effect is augmented by *meso*-inositol. The increased production of alcohol by this mold under the influence of thiamine was studied. A supply of the pyrimidine portion of the thiamine molecule increases alcohol production, but the thiazole portion alone has no such effect. *Penicillium digitatum* requires the thiazole moiety of thiamine; pyridoxine, pantothenate, and biotin are stimulatory (38). The effect of the last three compounds, and of biotin particularly, varies with the pH of the medium. At pH 6.5 and above they were much more stimulatory than at pH 3.0. Presumably the fungus synthesizes biotin and the other two compounds more readily in the acid environment or the metabolism is so altered that the compounds are not needed. *Piricularia oryzae* requires thiamine and biotin for growth and conidia production (39). Five isolates of *Sordaria fimicola* all require biotin and one of them thiamine also (40). Less biotin is required for prompt vegetative growth than for reproduction. In the presence of suboptimum amounts of biotin the number of perithecia is greatly reduced and their appearance is delayed. The ratio of biotin to the weight of the mycelium at the time of perithecial formation must be at least one to twenty million for completion of sexual reproduction (41). Several isolates of the cellulose decomposing fungus *Memnoniella* and the closely related *Stachybotrys* require biotin for growth and abundant sporulation occurs in the presence of this vitamin (42). Distinctly increased growth of *Pityrosporum ovale* is produced by thiamine, but in the presence of oxaloacetate and α-ketoglutaric acid thiamine is reported to be unnecessary (43).

Yeasts.—The requirements of about sixty yeasts for inositol, pantothenate, biotin, thiamine, pyridoxine, thiamine and pyridoxine together, and nicotinic acid were determined by Schultz & Atkin (44). The requirement for each yeast was summarized by a numerical designation termed the "bios requirement," which they consider a valuable complement to the present yeast classification methods. The necessity of providing an adequate supply of vitamins when determining yeast utilization of nitrogen sources, such as ammonium sulfate, urea, and asparagine, was emphasized by Wickerham (45). The statement that a particular yeast is incapable of utilizing ammonium sulfate, for example, is meaningless unless the vitamins required by that particular yeast are supplied. Pre-

vious yeast classifications based on such criteria really reflect the vitamin needs of the individual yeasts.

The Growth Factors

The past few years have witnessed striking advances in our knowledge of the structure and function of several vitamins. This is particularly true of the *L. casei* factor-folic acid-vitamin B_c substances (pteroylglutamic acid and related compounds), the vitamin B_6 group, biotin, and pantothenic acid. Much of this material has been reviewed quite recently, and the reader is referred particularly to the discussions of Woods (46), Woolley (47), Gunsalus (48), and Oser (49) in recent or current volumes of the *Annual Reviews*. The present discussion of the growth factors will be confined mainly to material which has not been covered in other articles. This method of treatment necessarily gives a somewhat fragmentary character to this section of the review, but it seems preferable to repetition.

Biotin.—The biological activity of oxybiotin, the oxygen analogue of biotin, apparently is an inherent property of the molecule and is not necessarily due to its conversion to biotin. Axelrod, Flinn & Hofmann (50) have advanced more evidence that *Saccharomyces cerevisiae* 139 utilizes this compound as such and does not convert it into biotin. Biotin could not be demonstrated in the cells of this yeast when grown on oxybiotin and at the same time approximately 100 per cent of the added oxybiotin was recovered.

A comparison of the growth promoting effect of *d*-desthiobiotin, *dl*-oxybiotin, and *d*-biotin showed no general agreement among nine species of butyl alcohol-producing anaerobes studied by Perlman (12). Approximately half of them responded to desthiobiotin about as readily as to biotin, but for others desthiobiotin showed little growth promoting effect. For a few of the clostridia oxybiotin was quite active; for others it possessed low activity. A curious effect was noted with mixtures of oxybiotin and biotin, and of desthiobiotin and biotin. These mixtures often showed activity corresponding to an equal quantity of biotin, though the oxybiotin or desthiobiotin alone showed low activity.

The biotin-like effect produced by some fatty acids and lipids in the metabolism of microorganisms continues to be a matter of

current interest. Substitution of oleic acid for biotin was reported by Williams & Feiger (26). In the presence of adequate amounts of oleic acid the biotin requirement of *Lactobacillus casei* is greatly lowered or entirely eliminated. Elaidic acid also exerts this effect. Some lipids and nonionic synthetic detergents are effective also in replacing biotin, the most stimulatory of them being oleates. Oleic acid does not induce biotin synthesis and evidently is not a precursor of biotin (26, 50a). The lipid stimulation is not nullified by avidin. Of the Tweens, those esterified with oleic acid are more effective as growth promoting agents than is oleic acid itself (28). Other Tweens, though inactive themselves, broaden the pH zone over which oleic acid activity may be observed. It is suggested that a function of biotin is to catalyze the synthesis of oleic acid (28) and also that biotin may be involved in cell permeability or surface action (26).

A vaccenic acid fraction from beef tallow showed some biotin-like activity for *L. casei*, but not for *L. arabinosus*, *S. cerevisiae*, or a hemolytic streptococcus (51). The existence of a fat-soluble material (FSF) capable of replacing biotin in the growth of *L. casei* and some other lactic acid bacteria was reported by Trager (52). The substance was obtained by acid or enzyme hydrolysis of the plasma of animals and man. FSF was not inactivated by avidin in microbiological tests with *L. casei* or in feeding experiments with chicks. Its properties, insofar as known, do not correspond to biotin and its analogues, or to oleic acid. It is believed, however, that there may be a relationship between FSF, oleic acid, and the utilization and function of biotin. In view of the results of others with oleic acid compounds, it is possible that FSF may contain combined oleic acid in a form available to the organism. Hofmann & Axelrod (53) found that in the ether soluble materials from human plasma the biotin-like activity for *Lactobacillus* occurs in the saponifiable part. Oleic acid on a weight basis has approximately the same activity, though it is not yet clear whether the plasma activity is due solely to oleic acid.

The interrelation between biotin and the fatty acid substances is an interesting one which needs to be further clarified. It may contribute materially to our knowledge of the metabolic role of biotin. The relationship between biotin and aspartic acid is discussed elsewere in this volume (48).

Nicotinic acid.—The question of bacterial utilization of various

pyridine derivatives has attracted attention since the first demonstrations of the physiological importance of nicotinic acid. A recent study of relative activities of a number of these compounds was made by Ellinger, Fraenkel & Abdel Kader (54) using several *Proteus* strains, dysentery bacilli and *L. arabinosus*. Most of the bacteria utilized nicotinamide and nicotinic acid about equally well. Other compounds were utilized to a much lower degree. In this category were quinolinic acid and β-picoline, which were utilized sparingly by all types. Of the other compounds supporting growth or acid production to a slight extent, nicotindiethylamide was utilized only by *Proteus* X13, and the monoethylamide by both *Proteus* X13 and *P. vulgaris*. Nicotinonitrile was reported to be utilized by *L. arabinosus*, a conclusion reached evidently on the basis of a small increase in titratible acidity although there was no visible growth. A series of other related compounds gave negative or essentially negative results. This included nicotinallylamide, several nicotinmethyl-cyclohexylamides, some nicotinphenyl- and benzylamides, trigonelline, and nicotinamide methochloride. This report is of interest, also, for a comparison of utilization by the rat, an insect, and the bacteria. In general, the results with the bacteria are in agreement with previous reports wherever the same compounds have been tested. Nicotinamide methochloride was found in the cells, but not in the liquid medium, of *P. vulgaris* after growth on nicotinic acid or nicotinamide.

Pteroylglutamic acid.—The comparative growth-promoting effect of compounds related to pteroylglutamic acid has been reported for several organisms by Lampen & Jones (55). For *L. arabinosus* 17-5, *p*-aminobenzoic acid, *p*-aminobenzoylglutamic acid, and pteroic acid are more active than pteroylglutamic acid on a molar basis, while pteroyltriglutamic acid and the glutamic acid polypeptide of *p*-aminobenzoic acid are less active. Kidder & Dewey (56) found that the protozoan ciliate *Tetrahymena geleii* W is capable of utilizing vitamin B_c conjugate (pteroylheptaglutamic acid). So far it is the only microorganism known to be capable of using this compound. Pteroyltriglutamic acid and pteroylglutamic acid were somewhat less active than vitamin B_c conjugate for *Tetrahymena*, while *p*-aminobenzoylglutamic acid, pteroic acid, xanthopterin, and a methyl pteridine were inactive, alone or in combination. The ability of *Tetrahymena geleii* W to utilize B_c conjugate may prove to be of value in microbiological assay since

most of the naturally occurring vitamin is in the conjugated form (56).

An interesting sidelight on the pteroylglutamic acid requirement of microorganisms is the substitution of thymine (5-methyl uracil) for this factor or for related factors from natural sources (*L. casei* factor, folic acid, vitamin B_c). The amount of thymine needed for equivalent growth is usually stated to be many times that of pteroylglutamic acid for *Streptococcus faecalis* and for most related streptococci, while for Lactobacillus species the replacement may be only partially effective (57, 58, 59). However, Hall (60) obtained more nearly equivalent responses by lengthening the period of incubation. Different interpretations of the thymine substitution have been dealt with in earlier articles. In a recent contribution Hall (60) favors the view that thymine may be a precursor of folic acid or that it participates in some alternative metabolic path. Rogers & Shive (60a) believe, as a result of experiments based on inhibition by methyl folic acid, that folic acid functions as a coenzyme in the biosynthesis of purines and thymine or their equivalents.

Aside from the pyrimidines, histidine has been reported to exert some folic acid-like activity after sterilization in the culture medium (61). The activity, it is believed, is due to an unidentified substance formed by reaction of histidine with some component of the medium. This substance appears to be neither folic acid nor pteroylglutamic acid.

p-Aminobenzoic acid.—With the demonstration that *p*-aminobenzoic acid (PAB) is part of the pteroylglutamic acid molecule it might be assumed that need for PAB is simply for construction of the larger molecule. However, there are indications that the PAB requirement can not be explained entirely on this basis. In several cases PAB has been found to be much more active than pteroylglutamic acid as a growth promoting agent; this is true for a mutant strain of *E. coli* (62) and for several lactic acid bacteria (55, 63). Also PAB on a molar basis was more active than *p*-aminobenzoylglutamic acid, pteroic acid, and pteroyltriglutamic acid (55). Lampen & Jones (55) suggest that pteroylglutamic acid, the purines, and thymine are products of enzyme systems in which PAB functions, while Shive & Roberts (64) on the basis of competitive metabolite growth inhibitions suggest PAB functions in the synthesis of methionine and certain purines.

An interesting reversal of the usual PAB sulfonamide relationship appeared in a mutant strain of *Neurospora crassa* studied by Emerson (65). This organism produced optimal growth only in the presence of sulfonamides, which were required at 35°C. and were stimulatory at 30°C. or lower. Within certain temperature limits (34°C. or over) PAB inhibited growth and the inhibition was competitively antagonized by sulfanilamide which, it seemed probable, was used as a metabolite. A double mutant was also obtained which required for growth both sulfonamides and PAB.

Vitamin B_6 group.—The connection of this group of compounds with amino acid metabolism is now clearly established. Recent contributions are reviewed in another article of this volume (48). The pyridoxal phosphate active as codecarboxylase evidently is not the 3-phosphate (66). The varying response of different microorganisms to pyridoxal phosphate, pyridoxamine phosphate, and their hydrolysis products is shown in the work of Rabinowitz & Snell (67). The phosphorylated compounds are much more active after acid hydrolysis for *Saccharomyces carlsbergensis* and *L. casei;* for *S. faecalis*, however, pyridoxamine phosphate is as active as the hydrolysis products.

Assay methods for the vitamin B_6 group were reviewed by Stokes (68), who described the *Neurospora* method in detail and discussed the sensitivity, accuracy, and reliability of several methods. The method for pyridoxal and pyridoxamine which uses *S. faecalis* as the test organism was modified by Rabinowitz & Snell (69) to eliminate earlier variable results. The apparent vitamin B_6 content of natural materials assayed by this method was less than that obtained with *S. carlsbergensis*, which responds to pyridoxine as well as to the two foregoing compounds.

Porphyrins.—Most cells synthesize their own iron porphyrins, but *Hemophilus influenzae* is unable to accomplish this in ordinary culture media. Granick & Gilder (70) studied the response of *Hemophilus* to various porphyrins. Iron protoporphyrin IX, which is required for growth of *H. influenzae*, can be replaced by protoporphyrin IX (containing no iron). Cells grown on the latter compound contain iron porphyrin and apparently insert iron into the protoporphyrin ring. A number of iron-free porphyrins which do not contain vinyl groups (in contrast to the protoporphyrin above), such as deutero-, hemato-, meso-, and coproporphyrin, usually do

not replace protoporphyrin for growth. Porphyrins lacking vinyl groups, after conversion to iron porphyrins, supported growth. When the propionic side chains of protoporphyrin and of iron protoporphyrin were esterified the compounds no longer supported growth. This work suggests that the vinyl groups assist in the insertion of iron into the porphyrin ring and the carboxyl of the propionic side chain attaches to the specific apoprotein to form the heme enzyme. Porphyrins lacking iron and vinyl groups inhibit growth of *H. influenzae* and the inhibition between a porphyrin and iron protoporphyrin is competitive.

Different strains of *H. parainfluenzae* show quantitative differences in their ability to synthesize heme. Their growth without heme depends in part on the properties of the medium which protect heme from oxidative breakdown (71). Agents or conditions that destroy peroxides produce growth stimulation by preventing heme destruction. Thus it happens that some strains which produce relatively low levels of heme appear to require it under certain conditions but not under others.

Fatty acids.—Dubos (72) found that an unidentified micrococcus grew abundantly on addition of 0.001 to 0.0001 per cent of oleic, linoleic, linolenic, or arachidonic acid to a mineral medium containing glucose as the sole source of carbon. Yeast extract and hydrolysates of casein and other protein were unable to take the place of the unsaturated fatty acids. Growth of a tubercle bacillus was enhanced by 0.001 to 0.01 per cent of a variety of fatty acids. When toxicity of fatty acids is encountered it can be reduced or abolished by esterification or by the addition of serum albumin. The albumin binds traces of unesterified fatty acid thus eliminating an inhibitory effect and permitting growth from a small inoculum (73).

It must be recognized, however, that the introduction of albumin into a medium containing a fatty acid compound may give rise to complications. Evidently many commercial serum albumin preparations contain lipase, which in turn releases enough fatty acid from a compound, such as Tween 80, to exceed the binding capacity of the albumin. Such a medium on standing gradually becomes bacteriostatic (74). This lipase effect, which in all probability occurs in many biological materials which might be added to culture media, can be eliminated by (*a*) the use of crystalline

albumin, (*b*) heating with appropriate precautions to avoid coagulation of the albumin, or (*c*) the use of 0.01 per cent sodium fluoride to inhibit the lipase.

The need of some lactic acid bacteria for oleic or related fatty acids and the interrelation between fatty acids and biotin have been given in preceding sections devoted to the lactic acid bacteria and to biotin.

Variation in Vitamin Requirement

The study of biochemical mutants produced by x-rays, ultraviolet light, and other means continues to be a fruitful field of investigation. Such mutants may differ from the parent or wild stock in a variety of ways. With respect to the vitamins, the mutants often are more exacting or show an added requirement not evident in the original culture. That is, they are now unable to form a compound which previously was synthesized from simpler ingredients in the cell environment. When it is possible to analyze them genetically, as in the pioneer studies of Beadle & Tatum with *Neurospora* mutants, the failure of synthesis appears to be related to the mutation of a single gene.

A summary of the work with *Neurospora* up to 1945 was published by Beadle & Tatum (75). From over 68,000 single-spore strains derived from cultures treated with x-rays, ultraviolet light, or neutron bombardment, more than 380 strains with altered nutritional requirements were obtained. Most of them on genetic study were found to differ by single genes from the original strains from which they were obtained. The added requirements were mostly for the B vitamins, amino acids, purine and pyrimidine bases, or some unidentified compounds. A requirement for fat-soluble vitamins was not encountered, though consistently searched for.

Mustard gas is also effective in inducing mutations. Horowitz and co-workers (76) exposed spores of *Neurospora* to mustard gas vapor and obtained 3.8 per cent mutants from 760 treated spores. The mutations involved both cultural and biochemical properties. Among the latter were strains requiring thiamine and *p*-aminobenzoic acid, as well as methionine, cystine, and other amino acids,

Irradiation of either spores or vegetative cells of *Bacillus subtilis* with x-rays or ultraviolet gave rise to a number of mutants with different requirements (77). From 4260 isolates 143 singly

deficient mutants were identified of which 33 required B vitamins and 106 required amino acids. A few isolates having multiple requirements were also obtained. Burkholder & Giles (77) are inclined to believe that the spores of *B. subtilis* are uninucleate and haploid.

A second radiation of mutants derived from a previous treatment is capable of producing further changes, such as added deficiencies in synthetic ability. Tatum (78) found that a second x-ray treatment of two *E. coli* mutants produced new mutants with other deficiencies. The original requirement persisted so that the new variants were characterized by an added deficiency.

In most cases, the change induced by the injury seems to involve complete inactivation of a process, such as the ability to accomplish a certain step in the synthesis of a vitamin. However, partial inactivations occur. Among recent reports is that of a *Neurospora* mutant which grew readily without riboflavin at 25°C. and lower, but required the preformed vitamin for growth above 28°C. (79). Beadle & Tatum (75) in their summary report that more than a dozen such "temperature sensitive" mutants were encountered.

Roepke & Mercer (80), using *E. coli*, studied the survival ratio after exposure to x-rays, the apparent cumulative effect of "nonlethal" hits, and other effects. Their results emphasize factors which must be considered in the quantitative study of x-ray-induced mutations. For example, from 4,420 colonies isolated from plates poured shortly after x-ray treatment, sixty-one growth-factor-requiring mutants were obtained; but when the x-rayed suspensions were held at 4°C. for from fifteen hours to eight days before plates were prepared, no mutants were found in a total of over one thousand colonies examined.

The work on induced mutants is significant with respect to the heredity units of the bacterial cell. The heritable deficiencies encountered in various species of bacteria are biochemically analogous to those in *Neurospora* associated with single gene mutations, so that it is assumed that bacteria possess genes or analogous entities. An interesting use of induced mutants is their application to the question of gene recombination in bacteria. Tatum & Lederberg (81) found that *E. coli* mutants with known deficiencies, when grown in mixed culture, gave rise to prototrophs. Thus a coli mutant characterized by inability to synthesize biotin, phenyl-

alanine, and cystine, as well as the ability to synthesize threonine, leucine, and thiamine, was grown in mixed culture with another coli mutant possessing the reverse characters. From this mixture there were obtained prototrophs able to synthesize all of the six foregoing compounds. Other nutritional types also appeared, such as strains able to synthesize five of the six compounds but deficient in the synthesis of the remaining one. The authors point out that the occurrence in mixed cultures of new types, which can be represented as recombinations of the characteristics of the original strains, suggests strongly that hybridization and segregation take place. They postulate a sexual phase in *E. coli*, a cell fusion allowing segregation of genes in new combinations in a single cell. Previous attempts to study this problem in bacteria by the use of somewhat similar technics had been made by several workers, but the results were not as definite as this more recent investigation, which appears to be one of the most significant of the many reports dealing with the question of a sexual phase in bacteria.

All of the foregoing work provides a quite logical explanation for the differences in vitamin and other nutritive requirements which are encountered in different bacteria and even among different isolates of the same species. Since the requirements of induced mutants correspond with those of organisms obtained directly from natural sources, it seems likely that spontaneous mutation followed by selection is responsible for the nutritive requirements of microorganisms as we encounter them in the course of the usual laboratory isolations.

Vitamin Synthesis and Cell Content of Vitamins

Effect of environment.—The amount of vitamin synthesized is often intimately connected with the composition of the culture medium and particularly with the metallic ion content. The production of riboflavin especially seems to be closely related to the amount of iron. High levels of riboflavin were produced by *Candida* yeasts when the iron content of the medium was carefully controlled (82). Apparently the amount of iron compatible with optimum riboflavin synthesis differs markedly with different species; often it is less than that associated with heaviest growth of the organisms. Amounts greater than 1.0 μg. of iron per ml. sharply decreased riboflavin formation by *Clostridium acetobutylicum* in corn mash (83). A bacteriological method for precipitation of re-

duced forms of riboflavin from certain culture media has been described (84).

Leviton (85) found that not only was riboflavin production lower in the case of *C. acetobutylicum*, but added riboflavin was actually destroyed in a medium containing small amounts of both ferrous ion and hydrogen peroxide. This report raises the interesting question whether relatively low production of a vitamin under certain conditions is due to decreased synthesis, as has often been assumed, or to accelerated destruction.

In a study of the effect of nitrogen and carbon sources upon riboflavin production by *Mycobacterium smegmatis*, Mayer & Rodbart (86) report that highest yields of flavin were obtained in the absence of organic nitrogen. The vitamin content of tubercle bacilli has been studied by Pope & Smith (87) and Bird (88). The effect of inhibitors on the yeast content of various B vitamins was studied by Eppright & Williams (89). Results differed with the vitamin and the inhibitor. Biotin was unique in that the amount in yeast grown in the presence of an inhibitor was never lower than that of the controls.

Adsorption.—Adsorption of vitamins from the environment may contribute also to the vitamin content of the cells of microorganisms. Van Lanen (90) found that nicotinic acid is taken up readily from the medium by both growing and fermenting yeasts; values as high as 1200 or more μg. per gm. of yeast were attained.

Pathways of synthesis.—Metabolite analogues or antagonists have been used in the attempt to clarify the processes of biosynthesis of vitamins by microorganisms. In studying the process of pantothenic acid formation by *E. coli*, Stansly & Schlosser (91) obtained evidence suggesting that pantoic acid or pantoate, rather than pantolactone, is the immediate precursor in the biosynthesis of pantothenic acid. The growth inhibition of several lactobacilli and of *E. coli* caused by cysteic acid is prevented competitively by aspartic acid. Shive, Ravel and associates (92, 93) believe that cysteic acid prevents pantothenic acid formation by blocking an enzyme of the system which decarboxylates aspartic acid to β-alanine.

To study the role of desthiobiotin as a precursor of biotin, Rogers & Shive (94) employed 2-oxo-4-imidazolidinecaproic acid, a compound which differs from desthiobiotin by lacking a methyl group. This compound at appropriate levels completely prevented

growth of *E. coli* and the inhibition was nullified by appropriate additions of desthiobiotin. Consideration of the data led to the belief that the *E. coli* biosynthesis of biotin must proceed through desthiobiotin as an intermediate and that no alternative mechanism is available to this organism.

Irradiation-induced mutants also have been used to study the pathways of vitamin synthesis, with some interesting results. In a series of such mutants it may happen that the synthetic ability has been blocked at different steps in the series of reactions necessary to form the vitamin. This, together with the fact that the intermediate compound, up to the point of blocking, may accumulate in the medium under certain conditions, has afforded an opportunity to study the hitherto quite obscure (in most cases) pathways by which vitamins and other compounds may be formed. Of several *Neurospora* mutants needing thiamine, one was unable to synthesize the thiazole moiety and another was unable to bring about coupling of the pyrimidine and thiazole entities to form thiamine (95). In two "cholineless" mutants of *Neurospora* studied by Horowitz (96), monomethylaminoethanol appears to be a normal intermediate in the synthesis of choline. Mitchell & Houlahan (97) obtained evidence indicating inosine as a precursor of adenine.

Another series of *Neurospora* mutants has been reported by Bonner & Beadle (98) with respect to the synthesis of nicotinic acid. Among five mutant strains requiring this vitamin there were three different genetic types with the synthesis blocked at different stages. From these cultures two intermediate substances were obtained in crystalline form which were believed to be oxypyridine carboxylic acids. A later study by Beadle *et al.* (99) has shown that kynurenine is an intermediate in the formation of nicotinic acid, preceding the oxypyridine carboxylic acids. Since kynurenine is a normal product of tryptophane metabolism this observation has a bearing on the tryptophane nicotinic acid relationship.

With mutants which require amino acids some comparable results have been secured, significant with respect to the steps in amino acid formation. These results are not presented here since they are outside the scope of this review. The methods used in these studies offer promising possibilities for further important results and should aid materially in elucidation of one of the most perplexing problems of vitamin study, that of how the vitamins

are formed by the cell from simpler substances. It is obvious, too, that the method has a wider scope of application than that of vitamin synthesis.

Massive Doses of Vitamin

In a study of the effect of large amounts of nicotinic acid and nicotinamide it was found (100) that concentrations up to 1,000 μg. per ml. had little effect on the rate of growth of a number of representative saprophytic and pathogenic types. Amounts of 3,000, 5,000, and 10,000 μg. per ml. caused increasingly retarded growth or, with the largest amount, at times complete inhibition in a simple culture medium of ammonium phosphate, glucose, and salts. Such concentrations are many thousand times the amount needed for optimal growth. The effect is evident whether or not the vitamin is needed (in small amounts) for growth of the organism. In the presence of casein digest the inhibitive effect of massive doses of the vitamin is less pronounced. Addition of 0.1 per cent yeast extract nullifies the inhibitive effect of 10,000 μg. per ml. of nicotinic acid or nicotinamide. That is, in the presence of this large amount of vitamin, the cells require yeast extract for growth. This effect of yeast extract could not be duplicated with a mixture of known vitamins and related compounds.

Growth Factor Destruction

Some of the B vitamins may be broken down and utilized as foodstuff for growth of microorganisms. Koser & Baird (101) found that 60 to practically 100 per cent of the nicotinic acid supplied as the only organic compound in a simple medium disappeared, as determined by chemical tests, during the growth of *Pseudomonas fluorescens* and related types from soil and of *Serratia marcescens* and allied species. Most of the cultures failed to grow when nicotinamide was substituted for nicotinic acid as the only organic compound, and all failed to grow in the presence of isonicotinic and picolinic acids. Nichol & Michaelis (102) showed that the decomposition of nicotinic acid by *Ps. fluorescens* is accompanied by oxygen consumption at the rate of about three moles of oxygen per mole of nicotinic acid. Benesch (103) reported that the mixed cecal flora of man destroyed about two-thirds of added nicotinic acid during anaerobic growth (under liquid paraffin) in peptone broth.

Thiamine was broken down by certain unidentified soil bacilli

when the vitamin was supplied as the only organic compound, but in the presence of a variety of readily available foodstuffs little or no thiamine disappeared (104).

Bacteria, apparently species of *Pseudomonas*, capable of utilizing pantothenate were obtained by Metzger (105) from soil and air. During growth for seventy-two hours 100 per cent of the pantothenate was destroyed when it was the only organic substrate present (0.1 per cent). In manometric studies the vitamin was oxidized to 50 per cent of completion. β-Alanine and pantoic acid were utilized also, but not pantoyl lactone. When cells were grown in the presence of casein hydrolyzate there was marked "sparing" of pantothenate.

The formation of trimethylamine [$(CH_3)_3N$] from choline, suggesting metabolism of the choline side chain with liberation of the $(CH_3)_3N$, was reported by Dyer & Wood (106). This was produced by *Shigella alkalescens* and by some species of *Proteus*, but not by *P. morganii* or *P. hydrophilus*, or by species of *Salmonella*, *Eberthella*, or *Shigella* species other than *S. alkalescens*. Trimethylamine could be detected in *S. alkalescens* cultures in twelve hours and reached 90 per cent of theoretical yield in twenty hours. Its appearance was accompanied by formation of organic acids and was inhibited strongly by the presence of glucose.

Some interesting observations on vitamin inactivation have been reported by McIlwain and associates (107, 108), who found that hemolytic streptococci, *C. diphtheriae*, and *P. morganii*, cause a marked decrease in pantothenate concentration with a loss of the growth-promoting powers of the pantothenate. This pantothenate inactivation is closely associated with glycolysis. Inactivation and synthesis of pantothenate by nonproliferating bacteria proceed with definite velocities which can be expressed as metabolic quotients, Qp, the mμM change in pantothenate per mg. dry weight per hour. The inactivation of pantothenate is independent of most nutrients but requires glucose. To a large extent it is also independent of growth. It thus appears that the interaction between an excess of pantothenate and various bacteria is "a much more vigorous one than would be anticipated from their minimal requirements." In one hour hemolytic streptococci inactivated ten to twenty-five times the pantothenate needed for their growth, and fifty to one hundred times the amount of pantothenate in their cells.

A similar relationship was found by McIlwain (109) between hemolytic streptococci and glutamine. During growth of the streptococci, glutamine disappeared from the medium and could not be detected in the cells in comparable quantity. The process could be reproduced with suspensions of nonproliferating cells in the presence of glucose. The decomposition yielded glutamic acid and ammonia.

These observations on the inactivation of growth factors by bacteria are of considerable interest from a number of standpoints. It is probable that effects of this sort are fairly common; indeed, some evidence has appeared in previous work in connection with the "wearing out" of vitamins or coenzymes. It is evident that our common method of expressing the relationship between a vitamin and microorganism does not take into account these phenomena. The statement of quantity of preformed vitamin needed for maximal growth, or measurement of the amount of growth in relation to amount of vitamin supplied, does not express adequately, or indeed, gives no clue at all to other interactions between the cells and excess vitamin.

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ANTIBIOTICS[1]

By John Hays Bailey and Chester J. Cavallito
Sterling-Winthrop Research Institute, Rensselaer, New York

The subject will be discussed under three general headings: the antibiotics per se, mode of action, and resistance development. The antibiotics will be discussed under their source, using an alphabetical rather than taxonomic order. Publications are disregarded that are abstracts of data or that present so little experimental data that critical evaluation is impossible.

Actinomyces

Most of the antibiotics produced by actinomyces have not been well characterized chemically and in only a few instances has the impulse to name the antibiotic substance been resisted. It may well be that at least some of the "new" antibiotics will be shown to be streptomycin or one of the other previously known substances.

Trussell *et al.* (1) report the production of antibiotic activity in shaken cultures of an unidentified actinomyces. On the basis of antibacterial activity and mouse protection tests, the authors believe that at least two antibiotics are produced by this organism. One is probably streptomycin; the other, on the basis of toxicity, is thought to be streptothricin-like.

Actidione, produced by a streptomycin-yielding strain of *Streptomyces griseus*, has been isolated and crystallized by Leach *et al.* (2) from streptomycin beers. Actidione differs from streptomycin in being highly antifungal but not antibacterial in action. It is moderately toxic for mice ($LD_{50}=150$ mg. per kg.). Recrystallized from amyl acetate, the colorless plates melted at 115°−116° C. and had an $[\alpha]_D^{25}=-2.8°$. It appears to be a diketone of the formula $C_{27}H_{24}N_2O_7$, molecular weight of 507. The dioxime disemicarbazone and the optically active biologically inactive diacetate derivatives have been prepared.

Actinorubin (m.p. 206°−214° C.), active against both gram positive and gram negative organisms (3), is produced by an *Actinomyces* ("A 103") resembling *Actinomyces erythreus*, *A. fradii*, *A. albosporus*, and *A. californicus*. Kelner & Morton (4) have reported that actinorubin does not allow development of resistant

[1] This review covers the period from January, 1947 to March, 1948.

organisms as readily as does streptomycin. Junowicz-Kocholaty & Kocholaty (5) proposed, on the basis of the helianthate of actinorubin, the empirical formula $C_6H_{14}N_3O_2$ or $C_9H_{22}N_5O_4$. Solutions withstand fifteen minutes' boiling at pH 6 to 7, give negative Molisch and Sakagouchi and positive biuret tests, and pass through a Visking membrane. A dilution unit of the relatively crude material is equal to about 0.18 μg. Morton (6) has reported the LD_{100} as 1.37 mg. per mouse, intraperitoneally, and considerable therapeutic efficiency in *Klebsiella pneumoniae* infections in mice.

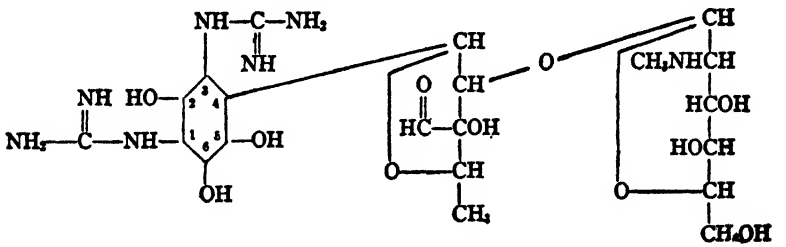
Ehrlich *et al.* (7) have isolated, from an as yet unidentified *Actinomyces*, chloromycetin which is active against both gram positive and gram negative organisms. Chloromycetin is a neutral compound soluble in water, alcohols, propylene glycol, and acetone. It crystallizes in elongated plates that melt at 149.7° – 150.7° C. and show an $[\alpha]_D^{25}$ of $-25.5°$. It contains 8.6 per cent nitrogen and 21.7 per cent chlorine. Aqueous solutions resist boiling for five minutes. This antibiotic is moderately toxic, the LD_{50} for 20 gm. mice being 3 mg. per mouse. Smadel & Jackson (8) have shown that this antibiotic possesses therapeutic power in rickettsial infections in mice. Three milligrams of chloromycetin *per os* protected all mice infected with lethal doses of *Rickettsia orientalis;* one-half that dose saved most of the infected animals. It has no effect on either variola or influenza A viruses, but some antiviral activity toward psittacosis virus.

Grisein, isolated from *Streptomyces griseus* by Reynolds *et al.* (9), is active against both gram positive and gram negative organisms but inactive toward fungi. The authors differentiate grisein from streptomycin on the basis of quantitative differences in antibacterial action of the two and the fact that the former is active against streptomycin-resistant organisms.

Lavendulin (3), a streptothricin-like antibiotic active against gram negative and gram positive organisms, has been partially purified by Junowicz-Kocholaty & Kocholaty (5) by the same techniques they used with actinorubin. Lavendulin closely resembles actinorubin in its antibacterial activity, being differentiated from it by showing much less activity against *Corynebacterium diphtheriae* and *Sarcina lutea.* Lavendulin is only slightly toxic (intraperitoneal LD_{100} is 0.5 mg. per mouse) and showed therapeutic activity *in vivo* in *Klebsiella pneumoniae* infections in mice (6).

A number of papers dealing with details of structure of strepto-

mycin have appeared during 1947 (10 to 16). The Merck group of investigators (14) suggested that the accompanying structure illustrates the points of connection of the various fragments of the molecule:



Carter *et al.* (10) suggested attachment of streptobiosamine to the 5 carbon of streptidine, although the 4 carbon is not entirely disregarded. Lemieux *et al.* (15) postulated that both glycosidic linkages in streptomycin are $\alpha-$L.

Dihydrostreptomycin, in which the free aldehyde group has been reduced to an alcohol, shows biological activity of the same order as streptomycin (11, 17). Oxidation of the aldehyde to a carboxyl group yields biologically inert streptomycinic acid (10).

A number of colorimetric assay methods for streptomycin have been described. Boxer *et al.* (18) outlined a procedure based upon formation of maltol from streptomycin and determination of the maltol by phenol reagent or ferric complex. Other colorimetric methods depend on formation of colored derivatives through the carbonyl group of streptomycin (19, 20).

A considerable number of articles have appeared dealing with the bacteriological assay of streptomycin. Only a few may be mentioned here. May *et al.* (16) found that in the bacteriological assay, culture medium, atmospheric conditions, nature of test organism, and size of inoculum all affected the end point. Herrell & Heilman (21) and Wolinsky & Steenken (22) also describe factors involved in the bacteriological assay of streptomycin.

An article of interest, but as yet difficult to evaluate as to significance, is that of Cohen (28) on the use of streptomycin and desoxyribonuclease in the study of the surface structure of T_2 bacteriophage of *E. coli*. Whereas streptomycin appears to react with phosphorylated nucleic acids to produce polymeric compounds, streptidine does not react.

Fitzgerald & Bernheim (24) showed that the oxidation of benzoic acid by certain Mycobacteria was more sensitive to inhibition by streptomycin than the oxidation of carbohydrates, fatty acids, and certain other substances. As the organism becomes more resistant to streptomycin, so does the oxidation of benzoic acid. Mycobacteria grown in media containing benzoic acid appear to be more resistant to streptomycin.

Many substances have been observed to react chemically with streptomycin to inactivate the antibiotic (25); other agents inhibit its activity *in vitro* (18, 11) by apparently competing for the site of adsorption of streptomycin. A most interesting observation of possible significance to the mechanism of action of the antibiotic is that of Rhymer *et al.* (26, 27), who show that lipositol has a specific antagonistic action toward streptomycin *in vitro*.

A very large literature has appeared during 1947 on the pharmacologic and chemotherapeutic activities of streptomycin. Space does not allow discussion of this work, but selected general references dealing with pharmacology and therapy (28, 29, 30), tuberculosis (31, 32, 33), tuberculous meningitis (34), tularemia (35), urinary tract infections (36), and granuloma inguinale (37) are cited.

Three new antibiotics bearing streptomycin as a portion of their names have been reported: streptomycin B, "residual form" of streptomycin, and streptomycin II. The latter substance was found by Johnstone & Waksman (38) in culture filtrates of an actinomyces resembling *A. griseolus* isolated from Bikini soil and designated *Streptomyces bikiensis*. The antibiotic substance exhibits approximately the same antibacterial activity as streptomycin, reacts to acid and glucose as does that antibiotic, and shows the same activity towards streptomycin-resistant strains of organisms as does streptomycin. No valid reason for distinguishing this substance from streptomycin has been advanced.

The "residual form" of streptomycin, reported by Hobby & Lenert (39), is contained in the fraction remaining after 400 to 500 μg. per mg. of crystalline salts of streptomycin have been prepared. The relatively pure streptomycin and the residual form show no difference in *in vitro* antibacterial activity toward most of the organisms tested. The "residual form" is at least five times more active than streptomycin when tested *in vitro* against *Eberthella*

typhi. In vivo, little difference can be demonstrated between the "residual form" and streptomycin.

Fried & Titus (40) isolated streptomycin B by chromatographic separation of streptomycin concentrates and fractional crystallizations of reineckates of the above chromatographic fractions. The reineckate (anhydrous) melts at 178°–179°C. (decomposition). Streptomycin B may be reduced by catalytic hydrogenation to dihydrostreptomycin B, with no loss in antibacterial activity. It probably contains streptose and a hexosamine; on degradation it has been shown to contain D-mannose (41). Streptomycin B is approximately one-fourth to one-eighth as active as streptomycin. Rake *et al.* (42), using a preparation of streptomycin B containing not more than 10 to 15 per cent of other streptomycins, found it to be about as active *in vitro* on a unit basis as streptomycin, but on a weight basis about one-third as active. Streptomycin B had essentially the same toxicity as streptomycin and *in vivo* was more effective than streptomycin, on a unit basis, in treating mice infected with *Salmonella schottmulleri*. On a weight basis, streptomycin was three times more effective than streptomycin B.

Streptolin, isolated from the culture filtrate of a species of streptomyces by Rivett & Peterson (43), is a basic antibiotic active against both gram positive and gram negative organisms. These authors have isolated streptolin as the crystalline helianthate (nitrogen 12.69 per cent, helianthate 63.6 per cent) melting with decomposition at 207°−211°C. The streptolin hydrochloride, prepared from the helianthate, is optically active ($[\alpha]_D^{28} = -22°$) and has 34,000 units per mg., using *E. coli* as the test organism. That streptolin preparations are contaminated with streptothricin has been shown by Peterson *et al.* (44) by absorption and elution methods. The antibiotics so obtained were compared with known samples of streptothricin, streptomycin, and streptolin for antibacterial action against six organisms. The results strongly indicate the presence of streptothricin in streptolin beers and clearly differentiate streptolin from streptomycin.

Junowicz-Kocholaty *et al.* (45) have isolated a sulfur-containing antibiotic, sulfactin, from culture filtrates of an organism resembling *Actinomyces roseus*. The material appears to be acidic and may be crystallized from ethanol-chloroform mixtures. The crystals melt over a wide range with decomposition, 245°−275°C.

The authors propose the empirical formula $C_{38}H_{56}N_{11}O_7S_4$, but consider the possibility of its being $C_{27}H_{40}O_5N_8S_3$. Sulfactin is insoluble in water but soluble in ethanol. It is primarily active against gram positive organisms. Morton (46) has shown that its antibacterial action is reduced one half in the presence of blood. Sulfactin is apparently effective in treating pneumococcus Type I infections in mice and possesses but a moderate toxicity.

Animal

Konikova *et al.* (47) have observed that bacteriostatic substances occur in certain animal organs and may be isolated by procedures similar to those used in the extraction of tyrothricin. The inhibition of bacteria such as *S. aureus*, *S. pryogenes*, *Bacillus brevis*, and *E. coli* by these extracts is said to proceed by the inhibition of dehydrogenase enzyme systems.

Zil'ber & Yakobson (48) have described procedures for separation of an antibiotic component from erythrocytes of a variety of animals and have named the substance erythrin. Erythrin is soluble in alcohol, acetone, and weakly alkaline aqueous solutions, but is insoluble in water. Heat does not destroy its activity. Diphtheria bacilli, *Bacillus brevis*, staphylococci, and streptococci are inhibited by approximately 0.03 mg. per cc. of erythrin, but common gram negative bacteria are only partially inhibited by 0.25 mg. per cc. *In vivo* protection of guinea pigs against diphtheria could be obtained only by simultaneous administration of drug and bacteria; erythrin appears to be poorly diffusible. Local treatment of typical diphtheria infections appeared to be fairly effective.

The increase at the onset of puberty of resistance of the scalp to infections by *Microsporum audouini* has been correlated by Rothman *et al.* (49, 50, 51) with an increase in concentration in the serum of low boiling, unsaturated fatty acids with fungicidal activity. The acids are unusual in being naturally occurring odd numbered carbon fatty acids; double bonds are usually in the 6, 7 position (52).

Nutini & Lynch (53) have prepared an 80 per cent alcohol-precipitated extract of beef-brain tissue. The extract proved satisfactory for both prophylactic and therapeutic treatment of mice infected with *S. aureus*.

Weissman & Graf (54) report on the action of calf-thymus histone in inhibiting oxygen uptake by *B. anthracis*.

Bacteria

The gram positive spore-forming rod, *Bacillus aerosporus*, has been reported by Ainsworth *et al.* (55) to yield an antibiotic, aerosporin, which is a base isolated much in the manner of streptomycin. The material was reported to be effective in protecting animals experimentally infected with *Haemophilus pertussis*, *Eberthella typhosa*, *Brucella bronchiseptica*, and *Escherichia coli*. The compound has the same order of chemotherapeutic activity against gram negative bacteria as penicillin has against the gram positive and is selective in its gram negative bacterial inhibition. The antibiotic does not induce resistance to it in bacteria and its action appears to be bactericidal. Aerosporin is probably identical with polymyxin, isolated by Stansly *et al.* (56) as a white, highly water-soluble hydrochloride. Polymyxin was found to be stable in the pH range of 2 to 7; it was not affected by proteolytic enzymes and is different from streptomycin, streptothricin, gramicidin S, tyrocidin, subtilin, and bacitracin. Formaldehyde treatment yielded a derivative that was soluble in sodium bisulfite and exhibited the original activity. Of sixty-four gram negative bacteria, fifty-six were sensitive to 0.5 to 16 μg. per cc. of the antibiotic hydrochloride; thirty-four gram positive bacteria were resistant to 64 μg. per cc. of the salt. Blood or changes in pH from 5 to 8 had little effect on activity. Polymyxin therapy was effective in mice infected with *Klebsiella pneumoniae* or *Pasteurella multocida*. The antibiotic was equally effective when given by subcutaneous or intravenous routes; the oral dose was sixty-four fold for the same effect. Polymyxin had no histamine-like action, 0.75 per cent solutions of the salt caused local skin irritation, and the LD_{50} for mice was approximately 300 mg. per kg., given subcutaneously. The formaldehyde-bisulfite derivative caused no irritation in 15 per cent solution and after daily subcutaneous administration of 100 mg. per kg., no toxic effects were observed after thirty days.

A second product appears to be present in the *B. polymyxa* fermentation liquors which demonstrates activity against gram positive bacteria (57). An agar diffusion assay method for polymyxin has been described using *E. coli* as the test organism (58). Proteolytic enzymes which inactivate subtilin are without effect on polymyxin (59).

Bacitracin, one of the *Bacillus subtilis* antibiotics, has been investigated relative to its pharmacological characteristics. Scudi &

Antopol (60) reported results from toxicity tests with rats and mice which show that toxicity is independent of antibiotic activity and may result from a histamine-like substance. Lethal results were obtained by oral administration with large doses only. Scudi *et al.* (61) believe that the antibiotic is destroyed in the gastrointestinal tract. Large doses administered by the intramuscular or the subcutaneous route resulted in appreciable blood levels in dogs even after seven hours. There was no apparent toxicity at 6,000 units per kg. Bacitracin is not freely diffusible and does not penetrate red blood cells, nor freely enter spinal fluid. Mice showed renal tubular necrosis. Eagle *et al.* (62) reported that bacitracin in rabbits and in man has a low renal clearance. The serum concentration of the antibiotic obtained after intravenous or intramuscular administration varied linearly with dosage. Urinary excretion was fairly constant for two to four hours after intramuscular injection. Meleney & Johnson (63) reported favorable response in surgical infections treated locally with bacitracin.

Gramicidin has been further treated with chemical agents to yield a modified antibiotic with altered activity and toxicity. Fraenkel-Conrat *et al.* (64) report that in alkaline solutions, gramicidin binds formaldehyde rapidly in amounts equivalent to its tryptophane content. The formaldehyde is believed to form methylol groups, probably on the nitrogen of the indole rings. In strong alkali, the formaldehyde may be removed from the gramicidin derivative without damage to the gramicidin molecule. Schales & Mann (65) treated gramicidin with acids, alkali, halogens, and other agents to yield derivatives which varied from 0 to 58 per cent of the gramicidin antibiotic activity and 2 to 87 per cent of the hemolytic action. The derivatives also showed qualitative differences in antibacterial activity. These investigators claimed that the conditions used in the formation of the methylol derivative would yield a modified gramicidin even in the absence of formaldehyde and that the methylol derivative differs from gramicidin in more than mere replacement of hydrogen atoms.

Consden *et al.* (66) carried out hydrolyses of gramicidin S under mild acid conditions and identified various di- and tri-peptides formed. From this they concluded that the antibiotic consisted of (-α-(L-valyl)-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-) occurring once or twice in a closed peptide chain, best formulated as the cyclo-penta or -deca peptide.

Mattick & Hirsch (67) have indicated that nisin, obtained from lactic acid streptococci, is different from diplococcin. Nisin has been obtained as needles in 16 to 20 mg. yield per l. of medium, an isolation yield of 20 per cent. The antibiotic does not diffuse through agar at pH 7 and is inactive against gram negative bacteria. At 4 mg. per cc. it had no effect on leucocyte motility nor on hemolysis of red cells; the LD_{50} in mice was 3.5 gm. per kg. Nisin protected mice with streptococcal infections and had some effect on tuberculosis in guinea pigs.

Young (68) observed the presence in *P. aeruginosa* culture broths of a heat-stable, water-soluble fluorescent antibiotic substance accompanying pyocyanine. Media which remained acid during the fermentation did not produce these antibiotic pigments. Burton *et al.* (69) described the amino acid requirements for pyocyanine formation by *P. aeruginosa* and found that L-leucine added to glycine or alanine in synthetic media markedly increased formation of the antibiotic.

Procedures have been described for the production of subtilin with surface (70) and submerged (71) cultures and purification methods given (72). Zinc has been claimed (73) as an essential element for growth and subtilin formation by *B. subtilis*.

A colorimetric assay method for tyrothricin has been described by Rittenberg *et al.* (74) which depends upon formation of a pink color with an acid solution of *p*-dimethylamine benzaldehyde.

Space does not permit discussing the fungal screening projects [Atkinson (75), Beal (76), Brian & Hemming (77), Wiken (78), Wilkins (79, 80)] published, nor the reports of activity of fungal culture filtrates or crude concentrates [Hauduroy & Rosset (81), Barta *et al.* (82)].

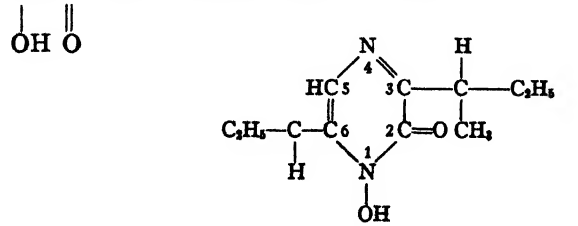
Fungi

Data are available on the fungistatic and bacteriostatic antibiotic isolated by Curtis & Grove (83) from the culture filtrate of a strain of the *Penicillium nigricans-janszewski* series. The compound is a red crystalline pigment that does not melt at 380°C. The molecule contains one methoxyl group but no nitrogen or halogen. The empirical formula appears to be $C_{10}H_8O_4$. The antibiotic is more active against fungi than against the gram positive organisms tested. In aqueous solutions the antibiotic is very unstable, more so as the pH approaches 7.

Kondo & Takahashi (84) reported the isolation of a crystalline antibiotic substance active against both gram positive and gram negative organisms from a fungus resembling *Penicillium spinulosum*. The antibiotic was obtained by solvent extraction of the acidified culture filtrate and crystallized from ether and acetone. The fine white needles so obtained melted at 183° − 185°C. This antibiotic is only slightly soluble in alcohols or acetic acid.

The antibacterial activity of a strain of *Penicillium tardum* reported by Wilkins & Harris (85) has been shown by Borodin *et al.* (86) to be due to a pale yellow optically active ($[\alpha]_D^{26} = -11.4°$ in alcohol) oil with the probable empirical formula of $C_{11}H_{16}O_3$. The antibiotic is stable between pH 2.0 and 8 but is decomposed at greater alkalinity with the formation of an acid and a neutral substance, suggesting, as the authors point out, the presence of a lactone structure. The antibiotic is active against gram positive organisms, *S. aureus* being inhibited in cup-plate tests by dilutions of 1:128,000 and *Streptococcus viridans* by dilutions of 1:8000. It is very sensitive to the presence of serum. The antibiotic is toxic.

Aspergillic acid has received renewed attention by Woodward (87) who studied the conditions for production of this compound by *Aspergillus flavus* grown in surface cultures. His strain of *A. flavus* also produced penicillin and the biologically inert hydroxyaspergillic acid as contaminants of the aspergillic acid. The determination of crude aspergillic acid in a culture filtrate is difficult, as a biological assay will show the effects of the contaminating penicillin, while the ultraviolet absorption technique also will give high values because of the hydroxyaspergillic acid content. Dutcher 88, 89) reported that the pale yellow rod-like crystals of aspergillic acid, $C_{12}H_{20}O_2N_2$, melt at 93°C., and in ethanol show an $[\alpha]_D^{25}$ of $+12° \pm 3°$. Dutcher pointed out the observed reactions of aspergillic acid point toward a hydroxamic acid type of grouping, —N—C—and to the structural formula:



Dutcher also prepared the optically active, biologically inactive

desoxyaspergillic acid $C_{12}H_{20}N_2O$. He believes that the antibacterial action of aspergillic acid is associated with the hydroxamic acid portion of the molecule. Synthetic hydroxamic acids showing antibacterial activity have been prepared.

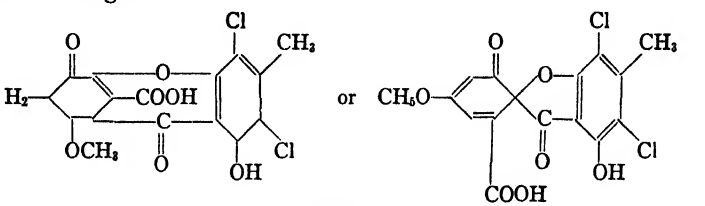
Robbins *et al.* (90) have isolated two antibiotics, biformin and biforminic acid, from *Polyporus biformis*. Biformin is extremely unstable; concentrated solutions rapidly lose their antibacterial activity, and evaporation to dryness of a chloroform solution of biformin results in loss of all antibiotic activity. Approximately 1 μg. of biformin per cc. is bacteriostatic for *E. coli* or *Mycobacterium phlei* but about nine times that concentration is required to inhibit the growth of *Bacillus mycoides*. Biformin is extremely toxic and without chemotherapeutic effect in staphylococcal or tubercular infections in mice.

Citrinin, first reported in 1931 (91) as a metabolic product of *Penicillium citrinum* and later as being produced by *Aspergillus terreus* (92) as well as members of the *A. candidus* group (93), has been shown by Pollock (94) to be produced by five additional species of Penicillia: *P. lividum* Westling, *P. phaeo-janthinellum* Biourge, *P. implicatum* Biourge, *P. chrzaszczi* Zaleski, and *P. citreo-sulfuratum* Biourge.

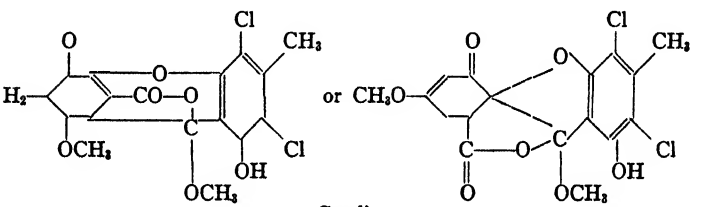
Fusarium orthoceras var. ennitinum has been shown by Gäumann *et al.* (95) to produce an antibiotic active against Mycobacteria and called by them enniatin. It was isolated from the mycelia as water-insoluble, colorless crystals, melting at 121°–122°C. and having a specific rotation (1 per cent solution in chloroform) of −92°. The proposed formula is $C_{24}H_{42}O_6N_2$. The antibiotic is slowly inactivated by acids but is rapidly destroyed by alkali. Enniatin is active primarily against gram positive organisms, completely inhibiting the growth of *S. aureus* in a dilution of 1:160,000, and *B. subtilis* at one-half that dilution. The Mycobacteria were inhibited by dilutions of the antibiotic ranging from 1:300,000 for *M. phlei* to 1:500,000 for certain human strains of the tubercle bacillus. Unfortunately, no data are available as to its toxicity or *in vivo* activity. Plattner & Nager (96) conclude, on the basis of antibacterial action and physical and chemical properties, that enniatin is identical with lateritiin I. They have isolated, from another *Fusarium*, two compounds closely related to lateritiin I, designated enniatin B and C. They apparently have the same empirical formulae: $C_{33}H_{55}O_6N_3$; "B" melts at 174°–176°C. and has an

$[\alpha]_D^{21} = -108°$; "C" melts at 152°–153°C. These compounds have about one-fourth the antibacterial activity of lateritiin I.

Erdin and its dextrorotary pseudoester geodin, produced by *Aspergillus terreus* Thom, were first described by Clutterbuck *et al.* (97, 98) and were of interest because of their chlorine content. Calam *et al.* (99) have shown that the optically inactive erdin is inert as an antibacterial agent whereas the optically active geodin (one form of the methyl ester of erdin) possesses appreciable activity against gram positive bacteria and only slight activity towards gram negative organisms. Two possible formulae are offered for erdin and geodin.

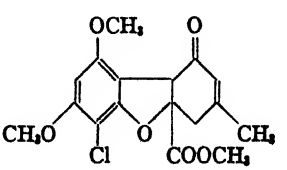


Erdin



Geodin

The "curling-factor," isolated by Brian *et al.* (100) and studied chemically by McGowan (101), has been shown by Grove & McGowan (102) to be identical with griseo-fulvin first described by Oxford *et al.* (103). The preliminary data on the curling-factor showed no halogens in the compound but later it was shown that chlorine accounted for about 9 per cent of the molecule. The formula that best fits the described reactions of griseo-fulvin is proposed as:

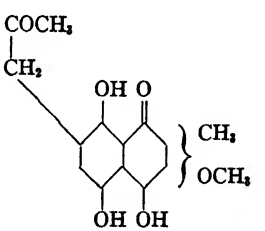


Brian *et al.* (104) have extended their observations on the fun-

gistatic action of glutinosin. Whereas this antibiotic is practically without action on gram positive organisms, more than half of the molds tested were inhibited by less than 1 μg. and only three required more than 10 μg. per cc. for complete inhibition in Weindling's medium.

Heatley *et al.* (105) have isolated a moderately active antibiotic substance, hirsutic acid, from culture filtrates of *Stereum hirsutum*. These workers have isolated the crystalline precursor of hirsutic acid N, designated hirsutic acid "C," and proposed the formula $C_{15}H_{20}O_4$. The precursor (hirsutic acid C) is without antibacterial action but its sodium salt is readily converted by *S. hirsutum* into the active antibiotic hirsutic acid N. Hirsutic acid N may be isolated from the mother liquor of "C" as crystals which do not melt (form a glass) and may be distilled in high vacuum at 125°C. with no loss in antibacterial activity. Treatment of the precursor "C" with dilute alkali converts it into hirsutic acid "A" which is moderately active against gram positive but inert toward gram negative organisms. Hirsutic acid N is more active than "A." Both are toxic.

Further chemical work by Arnstein & Cook (106) on javanicin and oxyjavanicin has led to a correction in the previously proposed structural formula for javanicin (107):



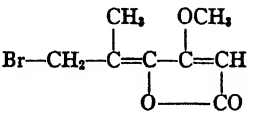
Javanicin, crystallized from ethanol, melts at 207.5°–208°C. (decomposition); oxyjavanicin, crystallized from chloroform or chloroform-petroleum ether, melts at 213°–214°C.

Cook *et al.* (108) report five new antibiotics produced by fusaria: lateritiin I ($C_{26}H_{46}O_7N_2$, melting point 121°–122°C.) produced by *F. lateritium*, lateritiin II ($C_{26}H_{46}O_7N_2$ melting point 125°C.) produced by "F.75" and probably *F. lateritium*, fructigenin ($C_{26}H_{44-46}O_7N_2$, melting point 129°C.) from *F. fructigenum*, sambucinin ($C_{24}H_{42}O_7N_2$, melting point 85°–86°C.) by *F. sambucinum*, and avenacein ($C_{25}H_{44}O_7N_2$, melting point 139°C.) from *F. avenaceum*.

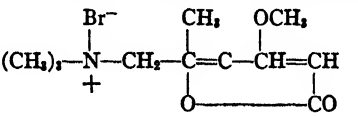
All of these compounds are neutral, optically active substances, sparingly soluble in water but very soluble in organic solvents and stable to heat and acids. They are inactivated in eighteen hours at room temperature by alkali at a pH of 10 to 11. Heating with hydrochloric or hydrobromic acids at 120°C. yields optically active crystalline bases, which retain all of the nitrogen of the original compound, and a crystalline acid of the formula $C_5H_{10}O_3$. With lateritiin I, α-hydroxyisovaleric acid is obtained. All of these antibiotics are active in high dilution against gram positive and acid-fast organisms. Lateritiin I completely inhibits the growth of three strains of *M. tuberculosis* in dilutions of 1:160,000 to 1:640,000.

Parasiticin (109), produced by *Aspergillus parasiticus*, has been shown by Arnstein & Cook (110) to be penicillin G. The authors had recognized this possibility in their earlier publications on parasiticin.

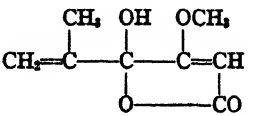
Birkinshaw *et al.* in 1936 (111) proposed a tetronic acid type of structure for penicillic acid. This structure has been confirmed by Raphael (112) by the synthesis of penicillic acid and its reduced inactive form, dihydropenicillic acid (113). By bromination of β-methoxy-γ-isopropylidine-$\Delta^{\alpha\beta}$-butenolid with N-bromsuccinimide,



was obtained. Treatment of this with trimethylamine gave



which on heating with magnesium oxide in water gave penicillic acid



On the basis of its ultraviolet light absorption pattern, Raphael concludes that in solution the antibiotic possesses the lactol structure rather than the carboxylic acid form.

DeSomer (114) has shown that treatment of *Penicillium notatum* spores with x-rays or ultraviolet light may produce mutants with greatly enhanced powers of penicillin production, but the majority of spores so treated are damaged. The enhancement of penicillin production was usually associated with a diminution or complete loss of certain other properties. This observation is confirmed by the work of Bonner (115) who showed that, although the best producing strains of *P. notatum* have been prepared by x-ray or ultraviolet irradiation of conidia, this treatment also can destroy the power of the organism to produce penicillin. Attempts to determine what step or steps in penicillin production were destroyed by the x-ray treatment by growing each of his fifty-five mutant strains in all combinations of two at a time with penicillin precursors were unsuccessful.

It had been shown that degradation of penicillin G yielded phenylacetic acid. This led Moyer & Coghill (116) to add this substance to the culture medium in the hope that the ratio of penicillin G:F would be increased. They found that this acid was toxic for the fungus and that the toxicity was a function of the pH of the fermentation mixture. By adding the acid after raising the pH or by delaying the addition until the fermentation had produced a favorable pH, they were able to increase the yield of penicillin about one-third. The G:F ratio, however, was not altered.

Koffler *et al.* (117) showed that one of the important roles of corn steep liquor in penicillin production was to supply the necessary minerals, particularly iron and soluble phosphate. They also showed that while more than 2 p.p.m. of copper inhibits the formation of penicillin, one-half this amount of iron overcomes the toxic action of copper.

One of the problems involved in penicillin production is the maintenance of the producing strain of the organism at a highly productive level. Whiffen & Savage (118) have shown that free sporulation led to mutation and the selection of low penicillin producing strains of the organism. Attempts to produce a superior penicillin producing strain by continuous isolation of high producing clones were unsuccessful. The workers were able, by using mycelia for propagation, to prevent "run down" of the producing strain for fifty transfers.

Not only has irradiation produced the best penicillin producing strains but this technique, as shown by Winsten & Spark (119),

has given new penicillins. These workers have demonstrated three new penicillins, S_1, S_2, and S_3, related to penicillin X, that are produced by *P. chrysogenum* Q176 as well as penicillins F, dihydro F, G, K, and X.

Considerable progress in elucidatingt he steps in penicillin production in synthetic media has resulted from the work of Jarvis & Johnson (120). They have shown that maximum penicillin production occurs under conditions that permit only a very slow growth-rate. The problem, then, is of setting conditions so that a maximum amount of growth as well as maximum penicillin formation can occur. The authors used a carbohydrate-acetate-lactate-ammonia type of synthetic medium and found pH 6.8 optimum for growth and pH 7.3 best for penicillin formation. The major factor affecting the fermentation rate was found to be the nature of the carbohydrate. By mixing glucose, which is rapidly used, with lactose (much more slowly utilized) in the ratio of 1:3 the conditions were set for sufficient mycelial growth as well as penicillin production. Ammonium ions are consumed rapidly during glucose utilization and slowly when lactose is used. The rapid loss in ammonium ion causes a fall in pH, tending to prevent penicillin formation. This fall in pH may be compensated for by supplying a fermentable anion that is used at about the same rate as the ammonium ion, a condition satisfied by acetate, except for the fact that it is rapidly consumed during lactose fermentation. Lactate is used slowly during penicillin formation and may be employed to overcome the loss in acetate. With such a medium the authors have obtained satisfactory yields of penicillin, and, with the addition of corn steep liquor, yields of from 500 to 700 units per cc.

The assay and separation of the various penicillins in commercial penicillin has received some attention. Two techniques are largely employed for the separation of component penicillins: the counter current distribution method of Craig and chromatography. Craig *et al.* (121) have shown that, in addition to the usual four penicillins, other as yet unidentified penicillins are present in the commercial product.

A method for simultaneous separation and determination of penicillins has been reported by Goodall & Levi (122). Ether is used as the mobile, and phosphate buffer supported on filter paper as the immobile, solvent. Ether solutions of the penicillins are run from reservoirs through the strips of buffer-containing filter paper,

depositing the penicillins along the strips. These are then placed on inoculated agar in petri dishes and incubated. Zones of inhibition will appear on the agar where the penicillins are located. By employing *S. aureus* and *B. subtilis* as test organisms, the approximate amount as well as the type of penicillins may be determined.

Brodersen (123, 124), during a study of the kinetics of acid inactivation of penicillin, has demonstrated a new component of commercial penicillin, "Y." This component is reported to be inert toward gram negative organisms but two-thirds as active as penicillin G for staphylococci. It possesses very little chloroform solubility and is much more stable to acid than penicillin G.

Welch and his co-workers (125) in the Food and Drug Administration were the first to show that a majority of amorphous penicillin samples contained a "factor" that enhances the action of amorphous penicillin. Its activity was reported as only an enhancing action for the therapeutic efficiency of penicillin in protecting mice infected with *E. typhi.* The "factor" is enhancing for all penicillins proportionally to their basic *in vivo* activity. Hobby *et al.* (126) have shown that the "factor" is present in penicillin beers and passes along with penicillin G during isolation and purification precedures. Degradation products of penicillin do not act as does the factor, although penicillinase-free clarase does. These workers have also shown that penicillin K may enhance crystalline penicillin G beyond that expected from summation of the two penicillins present. Similar observations have been reported by Rake *et al.* (127) in experimental syphilis in rabbits and by Groupé & Rake (128) with canary pox virus. Fischback *et al.* (129) attempted to isolate the "factor" and obtained *o*-hydroxyphenylacetic acid but state that this acid has no enhancement effect.

The relatively small amounts of penicillins other than G in commercial penicillins and the technical difficulties in their separation and purification has limited the amount of investigation done with them. Eagle studied their inactivation by serum (130), the blood levels obtained in animals and in man (131), and their therapeutic value in experimental pneumococcic and streptococcic infections (132). The fallacy of evaluation of an antibiotic by its *in vitro* bacteriostatic or bactericidal activity is well shown with these penicillins. As Eagle points out, if penicillin G be assigned the base value of 100 on its *in vitro* bactericidal powers, penicillins F, K, and X show, respectively, 60, 180, and 135 when Type I pneumococcus

is the assay organism and with *Streptococcus pyogenes* 75, 115, and 145. *In vivo*, however, penicillin K was found to be about one-tenth as active as the *in vitro* tests indicated. Furthermore, penicillin X was found to be significantly more active in the mice used than would be expected from the *in vitro* tests. Eagle believes this superiority to be related to the observation that penicillin X is more slowly inactivated by sera and gives higher and more prolonged blood levels than does G. The ineffectiveness of penicillin K, he believes, results from an apparently specific inactivation by serum.

Tompsett *et al.* (133) have shown that penicillins dihydro F, G, K, and X are bound in varying degrees by serum, particularly the albumin fraction, and noted a striking parallelism between their *in vitro* efficiency when tested in the presence of constant amounts of serum and their therapeutic activities. Thus penicillin X is the most effective of the four penicillins *in vivo;* it is least bound by serum and is least affected by the presence of serum in the assay. On the other hand, the *in vitro* activity of penicillin K is markedly reduced by serum; it is bound to a high degree by albumin and is the least active *in vivo* of the four penicillins. These workers, furthermore, have shown (134) that in man following the intramuscular injection of 300,000 units of the four penicillins (dihydro F, G, K, and X) blood levels are obtained that are (within the limits of the test used) essentially uniform as to magnitude and duration. They believe that the supposed rapid loss of penicillin K and the prolonged serum concentrations of penicillin X are artifacts brought about by differences in the binding action of the serum with the penicillins during actual assay procedures.

Space limitation prevents the discussion of the excellent pharmacologic work of Eagle & Newman (135), Price *et al.* (136), Jequier & Plotka (137) on the penicillins as well as the work of Fleming & Fish (138) and Macht (139, 140) on the effect of penicillin on coagulation of the blood.

A most interesting observation on the toxic action of penicillin has been made by Macht (141). White rats were trained to walk a tight rope, climb a vertical rope, and run a maze. With doses of 10,000 units of either amorphous or crystalline penicillin G, the time necessary to perform these feats was increased over the time needed by the same rat before the drug had been given. Similar, though more pronounced, effects were seen with streptomycin. The

depression was short lived and within three to four days the animals reacted as they did before treatment. Furthermore, a tolerance to the drug was developed, since after an animal had been used several times there no longer resulted an impairment of its performance.

Randall *et al.* (142) have reported that mice infected with *E. typhosa* and treated with small doses of penicillin show an increase in the fatality rate over the untreated, infected control animals. They have applied the term "hormesis" to this phenomenon and believe that it is an *in vivo* stimulation of growth of the infecting organism by subeffective doses of the antibiotic.

The effect of a single dose of penicillin compared with the same amount given in divided doses in the treatment of *Streptococcus pyogenes* infection in mice has been studied by Zubrod (143). The results are not validly comparable with the multiple dose data; nevertheless, a single dose of penicillin does show considerable therapeutic value.

Robbins *et al.* (144) have isolated an extremely unstable antibiotic, pleurotin, from *Pleurotus griseus*. It is optically active ($[\alpha]_D^{23} = -20°$), soluble in chloroform, ethanol, and ether, but only sparingly soluble in water. Exposure to light for a few hours completely destroys its antibacterial activity, as does alkali. The inactivated product is acidic. Pleurotin is active against gram positive organisms and slightly so against Mycobacteria and the gram negative bacilli.

Antibacterial substances have been obtained from several of the lichens. Marshak (145) isolated a crystalline acid (m.p. 191°–192°C.) from the lichen *Ramalina reticulata*. This acid, later shown by Marshak *et al.* (146) to be usnic acid, showed a slight amount of activity in combating tubercular infections in guinea pigs. It was inactive *in vitro* against gram negative organisms and showed only moderate antibacterial action against the gram positive and acid fast organisms. Stoll *et al.* (147) examined fifty-three species of lichens and obtained activity against *S. aureus* in twenty-two instances. The active compounds were D- and L-usnic, DL-usnic, D-protolichesterinic, vulpinic, and physodic acids, the most frequently encountered being usnic acid. These authors report (148) that usnic acid inhibits human strains of the tubercle bacillus in a dilution of 1:64,000 to 1:800,000 *in vitro*, but is inactive against gram negative organisms.

Plants

The synthesis of allyl-2-propene-1-thiolsulfinate, the antibiotic from *Allium sativum*, as well as a series of saturated alkyl thiolsulfinates has been described by Small *et al.* (149). The compounds are prepared by oxidation of the corresponding disulfides with an organic per-acid. The antibiotic activity of this class of compounds apparently depends upon the reactivity of the $\begin{matrix}-S-S-\\ \quad\downarrow\\ \quad O\end{matrix}$ structure toward sulfhydryl groups. The saturated alkyl derivatives are considerably more stable than the allyl derivatives and the higher alkyl members are less odoriferous. The thiolsulfinates show an increase in specificity of activity with increase of length of the alkyl group: the more lipophilic members showing, in general, greater activity against fungi and gram positive bacteria and less activity toward the gram negative group. The compounds are of possible value in the treatment of dermatophytic infections (150).

Stoll & Seebeck (151) have reported the isolation of alliin, the precursor of the garlic antibiotic. The compound was obtained from ethanol solution as colorless needles, m.p. 163°–165°C. (decomposition), $[\alpha]_D^{21}(H_2O) = +62.8°$. A molecular formula, $C_{12}H_{24}O_7N_2S_2$, was assigned to alliin, but later work (152) supports a formula of $C_6H_{11}O_3NS \cdot 1/2H_2O$. In a comprehensive article on the subject, Stoll & Seebeck (153) showed the structure of alliin to be $CH_2=CH-CH_2-\underset{\underset{O}{\downarrow}}{S}-CH_2-CHNH_2-COOH \cdot 1/2H_2O$. Hydrogenation of alliin yielded a dihydro-derivative, which, upon treatment with alliinase (the enzyme which cleaves alliin to the thiolsulfinate), yields the propyl thiolsulfinate in analogous manner to the formation of the allyl derivative from alliin. Oxidation of S-allyl-L-cysteine with hydrogen peroxide yielded an isomer of alliin with m.p. 146°–148°C., $[\alpha]_D^{20} = -12°(H_2O)$. This isomer also yielded the thiolsulfinate upon treatment with alliinase.

Cavallito & Kirchner (154) have reported the antibiotic of *Arctium minus* to contain an α-methylene butyrolactone type of structure, the prototype of which has been isolated from *Erythronium americanum* (155). The compound, isolated in this country, appears to be isomeric with that obtained by Abraham *et al.* (156) in England from *Arctium minus* and *Onopordon tauricum*.

Robbins *et al.* (157) have isolated an antibiotic, cassic acid,

from *Cassia reticulata*. The active principle is a yellow, crystalline, weakly acid compound, m.p. 330°C. ±. Cassic acid is inhibitory to several gram positive bacteria in from 0.004 to 0.008 mg. per cc. and is relatively inactive toward gram negative bacteria. Antibiotic principles other than cassic acid appear to be present in the leaves of this plant. The acid is relatively nontoxic in mice.

Raab (158, 159) has extended *in vitro* tests (160) with ergosterol and its activated vitamin form to show *in vivo* activity of these sterols in inhibiting the course of development of tuberculosis in guinea pigs and pulmonary tuberculosis in man. Charpy & Pichat (161) reported that cysteine antagonized the *in vivo* antitubercular activity and toxicity of vitamin D_2 in man. This may have some significance in suggesting the mechanism of antibiotic action of this vitamin.

Lieberman *et al.* (162) have reported synthesis of nordihydroguaiaretic acid, the bactericidal phenolic constituent of *Larrea divaricata*.

Plumbagol or 2-methyl-8-hydroxy-4-naphthoquinone (163), originally obtained from roots of *Plumbago europea* (164), has been reported to inhibit gram positive bacteria (165) in a concentration of 0.01 mg. per cc. and a number of human pathogenic fungi (166) at 0.025 mg. per cc. on solid media.

A large number of microorganisms have been tested by Holden *et al.* (167) for sensitivity toward protoanemonin. Activity is independent of age or inoculum size. The antibiotic has no effect on coli or staphylococcus bacteriophages or on influenza virus. Blood has some inactivating effect on the antibiotic.

Ivánovics & Horváth (168, 169) have reported isolation from seeds of the radish, *Raphanus sativus*, of a heat-stable, neutral oil, raphanin, inhibitory to *S. aureus* (0.035 mg. per cc.) and *E. coli* (0.2 mg. per cc.). Serum reduces activity. The syrupy liquid, b.p. 135°C. at 0.06 mm., has been assigned approximate molecular formulae of $C_{17}H_{26}O_4N_3S_5$ or $C_{17}H_{26}O_3N_3S_5$. The compound is neutral, soluble in water, and $[\alpha]_D^{20}$ (ethanol) $= -141°$. The antibiotic shows some resemblance to gliotoxin and shows $-SH$ groups after reduction and is inactivated by alkali or by hydrogen sulfide. The active principle is found in the plant in the form of an inactive precursor. The compound does not appear to have therapeutic promise. The antibiotic also has a blastocholine effect (inhibitory to seed-germination).

Irving *et al.* (170) have reported a few more properties of to-

matin preparations. George *et al.* (171) reported investigations for antibiotic activity among one hundred Indian medicinal plants. Alcoholic and aqueous extracts of many of the plants were inhibitory to *S. aureus* and some to *E. coli*, but they found that the plants known or claimed to be successful in bacterial chemotherapy were not necessarily those effective against the test organisms.

Mechanism of Action of Antibiotics

For the purposes of discussion, the mechanism of action of antibiotics will be considered with reference to three levels of organization of the microbial cell: (*a*) the action of the antibiotic upon the intact cell; (*b*) the effect of the agent upon an enzyme or enzyme systems; and (*c*) the nature of the reaction of the antibiotic with chemical functional groups of probable importance to the growth of the cell.

Most of the work of the past year has dealt with the effects of penicillin or streptomycin upon the intact bacterial cell. These investigations have often also dealt with the closely related problem of development of resistance to antibiotics by bacteria.

Gale (172) has applied a procedure for the determination of the free amino acid content of bacterial cells to the study of the action of penicillin on the assimilation of amino acids by *S. aureus* (234). The cells of *S. aureus* accumulate glutamic acid in steadily increasing concentrations when grown in media containing the amino acid. The addition of penicillin to such a medium results in a decreasing concentration of glutamic acid within the cells. Glutamic acid assimilation by normal washed cells is not affected by penicillin except in high concentration but is inhibited in cells which were grown in the presence of penicillin. The loss of assimilatory power can be correlated with loss of viability. The respiration, glucose oxidation and fermentation, and lysine assimilation of penicillin-treated cells are normal. The internal metabolism of glutamic acid is normal in such cells; but since the passage of glutamic acid across the cell wall is blocked by the penicillin treatment, the metabolism of this amino acid is limited by the internal concentration.

Frieden & Frazier (173) have reported on the effects of a number of substances upon the sensitivity of *S. aureus* to penicillin. Reduction in concentrations of magnesium or phosphate reduces the sensitivity to penicillin, but absence of glycine exerts an oppo-

site effect. Nicotinamide increases sensitivity to penicillin. Pyridoxine but not pyridoxal or pyridoxamine acts as a penicillin antagonist, but the effect appears to result from an *in vitro* inactivation of penicillin.

Yanagita & Suzuki (174) found that with both *S. aureus* and *E. coli* addition of penicillin at various stages of growth caused a decrease in bacterial numbers only at the transition from lag to log phase and during the log phase of the growth curve.

Schuler (175) has reported that penicillin as well as streptomycin act on the respiration of *E. coli* and staphylococci in the same manner; one obtains a latency period and an actively inhibitory period. Tyrocidin, gramicidin, gliotoxin, and clavacin do not act in this manner.

Dufrenoy & Pratt (176) have reported on some possible cytochemical mechanisms of penicillin action based upon observations of properties of areas on agar plates which show bacterial growth as compared with zones containing penicillin-inhibited microorganisms. On the basis of color reactions, lower sulfhydryl concentrations were found in zones of growth inhibition. An impairment of dehydrogenase activity was inferred. There may be some question as to the significance of differences in sulfhydryl concentrations between zones of inhibition and of growth since the growth zone obviously contains many more organisms than the inhibited areas. Pratt & Dufrenoy (177) described the use of dyes in following changes in cells induced by penicillin. Penicillin-treated *S. aureus* failed to apportion vacuolar material to daughter cells during cell division. Under the influence of penicillin, lipids were displaced from cells undergoing lysis and the liberated lipids were hydrolyzed into fatty acids.

Pratt & Dufrenoy (178) found that trace amounts of cobalt ions lower the effective penicillin threshold on test plates, and *in vivo* enhancement of penicillin action was also observed (179). Cobalt may act as a synergist toward a penicillin reaction with sulfhydryl groups.

Pandalai & George (180) suggest that penicillin interferes in phases where nucleic acid plays a part in cellular processes. Nucleic acid is claimed to antagonize the bacteriostatic action of penicillin, and penicillin bacteriostasis may also be reversed by nucleic acid. Gros & Macheboeuf (181) report that penicillin inhibits hydrolysis

of adenosinetriphosphoric acid with nonproliferating suspensions of *Clostridium sporogenes.*

Krampitz & Werkman (182) found that only impure preparations of penicillin inhibited dismutation of pyruvic acid. Pure penicillin G was reported to interfere with the dissimilation of ribonucleic acid and consequently with its assimilation during growth. Both intact cells and enzyme preparations of *S. aureus* failed to show any effect of penicillin on enzymes of intermediate products of glucose metabolism. This confirms earlier observations of Schuler (183).

Strauss (184) has found that bacteriostatic concentrations of streptomycin produced morphological changes in some gram negative bacilli but not in others; gram positive cocci were only slightly affected and bacilli were unaffected. Streptomycin was found to be bactericidal for *E. coli* in the resting state, the effect being related to size of inoculum and concentration of antibiotic. Exposure of susceptible strains of *E. coli* in the resting state to streptomycin did not result in increased resistance.

Results of a study by Geiger (185) on the effects of streptomycin on *E. coli* metabolism suggest that an unidentified intermediate formed in the course of oxidation of fumarate by this organism is a necessary part of the amino acid metabolizing system and that the metabolism of the amino acids in the presence of this intermediate is prevented by streptomycin. The hypothetical intermediate was also formed from the oxidation of malate, succinate, oxalacetate, glucose, lactate, and glycerol, but not pyruvate. The presence of phosphate or co-carboxylase aided in the formation of the intermediate from fumarate.

Lenert & Hobby (186) believe that the action of streptomycin is bacteriostatic rather than bactericidal. Sensitivity of an organism to streptomycin was found to be influenced by a number of factors. Berkman, Henry & Housewright (187, 188) found that a variety of unrelated substances antagonized the inhibitory effects of streptomycin. These agents may act by merely interfering with the adsorption of streptomycin at its site of action. Rhymer & Wallace (189) have reported an antagonistic effect on the action of streptomycin by plant proteins and extracts of brain tissue (liositol).

Benham (190) observed that addition of 1,000 units per cc. of

streptomycin to a system containing *E. typhosa* in which endogenous respiration was proceeding resulted in an immediate increase in rate of oxygen uptake. After two hours, the rate decreased until, at six hours, it was less than that of controls.

Hirsch & Dosdogru (191) report that both streptomycin and penicillin halt respiration of staphylococci after a latency period. Two types of action of penicillin are reported; at 0.05 to 0.1 units per cc. there is a long latency and then a rapid decrease in respiration which accompanies disintegration of the cocci ("degenerative effect"); at 1 to 1,000 units per cc. a cessation of respiration results after a shorter latency period which corresponds to the bacteriostatic effect. Autolysis does not occur after addition of the larger quantities. (It may be that larger quantities inhibited autolytic enzymes.) This is at variance with some earlier work.

Massart *et al.* (192) postulate that streptomycin and acridines or basic dyes in general interfere with metabolism of nucleoproteins by displacing physiologically active ions, especially hydrogen, from electro-adsorption complexes.

Relatively little work has been done on the mechanism of action of antibiotics other than penicillin or streptomycin. Hoffmann-Ostenhof and co-workers (193) report studies with quinones, which indicate that these compounds may inhibit growth by a number of mechanisms. No correlation could be made between antibacterial activity and ability of quinones or clavacin to inhibit urease activity (194). Antibacterial action was also found to be inversely related to the ability of the agents to inhibit blood catalase (195). The mechanism of action of quinones may vary with their structure; for example, the type of reaction which occurs between quinones and sulfhydryl compounds may vary with the nature of substituents on the quinones.

Rinderknecht *et al.* (196) have observed the effects of cysteine on natural and synthetically related antibiotics of the unsaturated carbonyl and lactone types. Most of the active compounds appeared to be sensitive to the sulfhydryl group of cysteine, but details of reaction mechanisms were not investigated.

Kojic acid, a weak antibiotic, appears to interfere with oxidative mechanisms, particularly involving amino acids (197). Whether this has any significance to its mechanism of antibiotic action is unknown.

Karrer & Viscontini (198) have observed some inhibitory effect by clavacin and a number of other organic compounds on the action of co-carboxylase on pyruvic acid.

Brief mention is made by Mitchell & Crowe (199) of observations of electron micrographs of normal and tyrocidine-lysed streptococci, showing that tyrothricin causes an actual rupture or partial solution of the cell wall of *S. faecalis*.

The aforementioned observations illustrate how nebulous our present state of knowledge is with reference to mode of action of antibiotics. The work of Gale and co-workers with penicillin and the earlier work of Hotchkiss with tyrocidine and gramicidin have been fairly fruitful approaches to the study of the effect of antibiotics upon intact cells. This should stimulate further study of the characteristics of bacterial cell membranes. Very little definite success has been achieved in attempting to associate the action of antibiotics with inhibition of specific enzymes or enzyme systems. As to the question of specific functional groups in cells which are reactive toward antibiotics, the reviewers feel that the marked reactivity of many antibiotics toward sulfhydryl groups and the known importance of sulfhydryl groups to growth processes is more than coincidental. Variations in reactivity of model thiol compounds toward various antibiotics may be an indication of the specificity of the antibiotic-cellular sulfhydryl inhibition mechanisms (200). Differences in reactivity of antibiotics toward sulfhydryl type compounds should lead one to expect differences in the manifestation of inhibitory mechanisms at the enzyme or enzyme system level, or the intact cell level of investigation. Some antibiotics such as the polypeptide types and aspergillic acid are not sulfhydryl reactive and may operate as surface active agents or by tying up trace elements (aspergillic acid) or by competition with metabolites by adsorptive displacement. The great diversity of chemical structures encountered among antibiotics has been quoted to reduce the possibility that their antibacterial action is due to a single mechanism (201). Again, speaking only of the level of mechanism of action involving reaction of functional groups, the reviewers feel that the sulfhydryl inhibition theory is the only common denominator for a large group of antibiotics of heterogeneous chemical structure. An alternative to such a mechanism would be to postulate specific metabolite-antimetabolite relationships for the many peculiar compounds. To our knowledge, this has not yet been done definitely with even one antibiotic.

Resistance to Antibiotics

For the purpose of this review, the term "resistance" is defined as a decrease in the susceptibility of a naturally susceptible organism to a given antibiotic, and "induced resistance" as that brought about by repeated subculturing of the organism in increasing concentrations of the antibiotic.

The frequency with which resistant organisms are isolated from patients undergoing treatment with streptomycin is the chief defect of that antibiotic; penicillin-resistant organisms are encountered less frequently but nevertheless are an important problem in the clinical use of this antibiotic. There are two theories as to the origin of resistant organisms. One theory attempts to explain their occurrence as an effect of the antibiotic on the bacterium, resulting in a metabolism so altered that the organism can grow in a normally inhibitory concentration of the antibiotic; the other, proposed by Demerec (202) and Luria (203), may be termed the genetic theory. It holds that within any bacterial population individuals will be found in varying numbers that possess varying degrees of susceptibility to a given antibiotic. The susceptibility of a given strain of an organism to an antibiotic is, then, the antibiotic effect on the vast majority of the organisms in the test. There are, however, a few organisms present that are less susceptible than the majority. In the presence of the antibiotic the majority of the population is inhibited and only those few resistant forms or mutants can develop.

The prediction (204) that with current methods of therapy penicillin and streptomycin resistance is less likely to be encountered has not been borne out. Barber (205) isolated staphylococci from two hundred patients admitted to surgical wards between April and November, 1946 and determined their penicillin susceptibility; twenty-five were resistant to at least 10 units per cc. of that antibiotic. Between February and June, 1947, of one hundred similar isolations, thirty-eight organisms grew in the presence of 10 units of penicillin per cc. These data are in keeping with those of North & Christie (206) who found that thirteen of thirty-one strains from patients having received penicillin were resistant to 10 units per cc. of that drug.

Resistance development *in vivo* is more striking with streptomycin. Youmans & Karlson (207) found the majority of strains of human and bovine tubercle bacilli isolated from patients before streptomycin therapy to be sensitive to 1.56 μg. per cc. or less;

after several months' treatment organisms isolated from the same patients were resistant to several milligrams of the drug. Resistance to streptomycin occurs as readily in gram negative organisms. Finland *et al.* (208) reported isolating a strain of *E. coli* from the urine of a patient on admission, this isolate being susceptible to 25 μg. of streptomycin per cc. The patient was given about 6 gm. of streptomycin. The next day the *E. coli* isolated from the urine was resistant to 50,000 μg. per cc. Alexander & Leidy (209) isolated a type B *Hemophilus influenzae* from the spinal fluid that was inhibited by 1.2 μg. per cc. of the antibiotic; within twenty-four hours after streptomycin therapy was begun the spinal fluid yielded a pure culture of *H. influenzae* that grew well in 1,000 μg. per cc. of the drug.

Resistant organisms in stock culture collections have been described by several workers. Segalove (210) has reported that among twenty-one coagulase positive *S. aureus* strains, fifteen of which produced enterotoxin, eight (all enterotoxic) required 100 or more units per cc. of penicillin to prevent their growth. Klein (211) studied six strains of *S. aureus*, one of *S. albus*, and one of *Streptococcus viridans* for resistance to penicillin, and one strain of *E. coli*, one of *Proteus vulgaris*, as well as the above staphylococci for streptomycin resistant individuals by inoculating one hundred tubes of medium containing inhibitory concentrations of the antibiotics with large inocula of the organisms being tested. No organisms resistant to penicillin were found in the original cultures, whereas all cultures contained individuals resistant to streptomycin. While this technique may be open to criticism as a measure of resistance, the data obtained clearly show that streptomycin-resistant organisms are more frequently encountered than are those resistant to penicillin.

Miller & Bohnhoff (212, 213, 214) have studied induced resistance to both penicillin and streptomycin in meningococci. All of the eighteen strains studied could be trained to grow in many times the original inhibitory concentration of both antibiotics. They found that resistance appeared more rapidly to streptomycin than to penicillin and believe the antibiotic acts to "select" naturally occurring resistant organisms present in the original culture. Seligmann & Wassermann (215) studied induced resistance to several organisms and found the development of resistance to streptomycin to be very rapid. Their strain of *S. aureus* increased in resistance from 1 μg. per cc. to more than 50,000 μg. per cc. in but

seven transfers. These authors suggest that the resistant strains have an altered "lock" the "streptomycin key" no longer fits. The *in vitro* resistance development by tubercle bacilli to streptomycin has been studied by Williston & Youmans (216). Nine strains increased in resistance at least one thousandfold during fifty-two to one hundred twenty days whereas nine other strains showed a less marked increase in resistance. Three strains showed no significant difference in susceptibility after eighty-five days' exposure to the antibiotic.

Klimek *et al.* (217) studied the resistance development of *S. aureus* 209P to a variety of antibiotics and showed that this organism could be adapted to grow in 4 mg. per cc. of penicillin, an increase in resistance over the sensitive parent of eighty-thousand times. This organism also developed marked resistance to streptomycin and the active principle of *Asarum canadense* but not, to any marked extent, to gliotoxin, aspergillic acid, pyocyanine, mercuric chloride, or the antibacterial principles of *Allium sativum* or *Arctium minus*. Bellamy & Klimek (218) found that *Streptococcus mastiditis*, *S. faecalis*, and *Clostridium perfringens* showed little decrease in susceptibility to penicillin during twenty to forty transfers in penicillin broth.

There is good agreement in the work reported on resistance that the resistant strains, either naturally occurring or induced, retain their resistance through many generations. Alexander & Leidy (209) passed their resistant *H. influenzae* strains through one hundred transfers in streptomycin-free broth with no loss in resistance. They also have been able to follow one of the patients in whom a resistant strain appeared for over a year; the patient still harbors resistant *H. influenzae* organisms. Williston & Youmans (216) have shown that the resistance of their tubercle bacilli to streptomycin persisted for at least eleven months, data in agreement with those of Middlebrook & Yegian (219). Murray *et al.* (220) passed thirteen strains of streptomycin-resistant gram negative organisms through one hundred transfers in streptomycin-free broth with no loss in resistance except in two strains where considerable numbers of susceptible cells developed. Streptomycin-resistant tubercle bacilli used to infect guinea pigs were found by Karlson *et al.* (221) to show the same resistance when isolated from the animals ten weeks or more later. On the other hand, Spink & Ferris (222) and Klimek *et al.* (217) are somewhat in disagreement with the above observations. Spink & Ferris believe that the resistance developed

in vivo differs from induced resistance, the first being more permanent and due to the production of penicillinase by the resistant organism. Klimek *et al.* showed that resistance could be lost unless "fixed" in the organism by maintaining it for a time in the presence of the antibiotic.

In general, organisms developing only slight degrees of resistance show little or no morphologic differences from the susceptible organism; when resistance is increased or marked, striking morphological changes have been reported. These changes are not a new observation, nor are they a function of the antibiotic. Walker & Murray (223) in 1904 noted that various gram negative organisms when grown in a medium containing 0.2 per cent methyl violet presented a profoundly altered morphology; *E. coli* appeared as filamentous rods so long that they often exceeded in length the diameter of the field of a one-twelfth immersion lens. This description fits as well the appearance of a penicillin- or streptomycin-resistant *E. coli.* Gardner (224) reports that resistant *Clostridium perfringens* cells attain lengths ten times greater than normal in the presence of penicillin; staphylococci show spherical enlargement and spindle formation. Bahn *et al.* (225) rendered five strains of gonococci resistant to penicillin and found all strains to consist of pleomorphic cells of increased size. Hall & Spink (226) noted that cells comprising a colony from a highly resistant *Brucella abortus* strain were "tiny amorphous coccoid forms staining faintly red and large dark red coccoid forms." Klimek *et al.* (217) found that *S. aureus* growing in 1 mg. per cc. of penicillin was rod-like in shape and gram negative. The morphology and tinctorial reaction reverted to that typical for *S. aureus* when the resistant strain was subcultured repeatedly in penicillin-free broth. With continued cultivation in penicillin broth the altered morphology and staining reaction apparently became fixed. These findings have been substantiated by Gale & Rodwell (227) who found no change in morphology or staining reaction in their *S. aureus* growing in 2,000 units per cc. of penicillin, but when its resistance had been increased to 6,000 units per cc. they found it to be highly pleomorphic and gram negative.

Isolated reports of biological differences between resistant and sensitive strains of the same organism have appeared, but the majority of workers report no such differences. There is general agreement that resistant organisms grow more slowly than do the parent as noted by Frieden *et al.* (228), Hall & Spink (226), Seligmann &

Wassermann (215), Klimek *et al.* (217), Murray *et al.* (229), and Schoenbach & Chandler (230). Penicillin-resistant gonococci have been reported by Bahn *et al.* (225) to ferment glucose at a slower rate than the sensitive parent strains. Murray *et al.* (229) found two resistant *K. pneumoniae* strains to have lost the power of fermenting saccharose, of using citrate, and of producing acetylmethylcarbinol.

The extremely resistant *S. aureus* studied by Bellamy & Klimek (218) was found to have lost the ability to grow anaerobically and they suggested that penicillin interfered with one or more essential components of the anaerobic energy mechanism of the susceptible organism. These authors further showed (231) that the resistant organism had acquired the ability to synthesize nicotinic acid, one of the essential vitamins for the growth of *S. aureus*, and to produce penicillinase, but had lost the power to grow in 6.5 per cent sodium chloride, to reduce nitrates, and to produce acid in saccharose, lactose, or mannitol. It retained its ability to ferment glucose.

The amino acid metabolism of penicillin-resistant staphylococci and their sensitive parent strains has been studied by Gale & Rodwell (227), Gale (172, 232), and Taylor (233). They have shown that gram positive cocci are able to assimilate and store certain amino acids in the free state within the cell prior to utilization and that penicillin added to growing cultures of *S. aureus* produces an impairment of the ability of this organism to store glutamic acid (234). Furthermore, Gale has shown (235) that as *S. aureus* becomes resistant it loses the ability to store glutamic acid, the loss being proportional to the degree of resistance developed, until a point is reached where the resistant *S. aureus* no longer can store glutamic acid (227). These resistant strains are gram negative. Gale & Rodwell (227) studied the amino acid metabolism of two such staphylococci and found that they were able to grow and synthesize amino acids in a medium composed of salts, ammonium sulfate, glucose, cysteine, and thiamine. Delayed and submaximal growth occurred when cysteine was omitted from the medium.

In contrast to penicillin, resistance to streptomycin is frequently accompanied by more obvious changes in the activities of the organism. According to Bernheim & Fitzgerald (236) oxidation of benzoic acid by various strains of *M. tuberculosis* is inhibited by streptomycin, 10 μg. of the antibiotic being sufficient to inhibit the oxidation of 1 mg. of the acid. They have shown that such an or-

ganism (ATCC #607), made resistant by passage in Long's medium containing streptomycin, will oxidize benzoic acid in the presence of 100 μg. of that antibiotic.

Changes in colony characteristics of organisms grown in the presence of streptomycin have been reported; the atypical colonies may or may not show altered metabolic activities. Hall & Spink (226) found two colony types in a resistant strain of *B. abortus*. One was large; the organisms in this type of colony grew in the presence of 50,000 μg. per cc. of streptomycin and were agglutinated by anti-*Brucella abortus* serum. These organisms, however, did not ferment any of the sugars normally fermented by *B. abortus*. The small colony was composed of organisms typical of sensitive *B. abortus* in fermentation and serological reactions but could grow in 7,500 μg. per cc. of streptomycin. These organisms died out readily unless heavy inocula were employed, suggesting a decrease in the production of some essential metabolite. Organisms from both colony types were stimulated in growth by subinhibitory concentrations of streptomycin, the members of small type colonies more so than those from the large.

Miller & Bohnhoff (213) have reported two colony types of resistant variants in all of the eighteen strains of meningococci studied. Large inocula (1 to 2×10^{10} organisms) were sown on agar plates containing increasing concentrations of streptomycin up to 4,000 μg. per cc. Plates containing at least 40 μg. per cc. of the antibiotic showed a few yellowish colonies 3 to 5 mm. in diameter. These resistant organisms, designated "variant A," grow in 10,000 μg. per cc. of streptomycin and have retained their resistance through weekly transfers in streptomycin-free medium for a year. Passage of this variant through mice did not cause a decrease in their resistance to streptomycin. The other variant, "B," appeared on plates containing 40 μg. or more per cc. of the antibiotic. The colony size and color depended upon the concentration of the drug in which they grew. This variant grew in 5,000 μg. per cc. of streptomycin and required not less than 5 μg. per cc. of the antibiotic for growth if large inocula were used. When small numbers were inoculated 100 to 400 μg. per cc. of streptomycin were required to give abundant growth. Streptomycin inactivated with either hydroxylamine or cysteine did not allow growth of this variant. It retained its type specificity, gram-staining characteristics, and fermented glucose and maltose if the test medium contained 100 μg. per cc. of streptomycin. Variant "B," unlike "A," is less viru-

lent than the parent culture; it will not kill mice unless they receive streptomycin.

Resistant organisms that require streptomycin for their growth are not confined to the meningococci. Kushnick *et al.* (237) isolated a variant from a streptomycin-resistant strain of *Bacillus subtilis* that requires a concentration of 150 to 300 μg. per cc. of that antibiotic for its growth. This variant grows only anaerobically. The susceptible parent and the streptomycin-requiring variant were tested for their ability to grow in an ammonium chloride synthetic medium to which various sources of carbon were added. The susceptible parent strain failed to grow in any of these media, but the streptomycin-requiring variant grew equally well in those containing streptomycin with glucose or glucose and sodium glutamate. Glucose could not replace streptomycin for the growth of this variant. Paine & Finland (238) have isolated streptomycin requiring variants from strains of *E. coli*, *S. aureus*, *P. aeruginosa*, and *P. morgani*.

Certain facts in the data on resistance are especially suggestive in their implications. Resistance to penicillin and streptomycin is widely distributed among all bacteria and is apparently more pronounced against streptomycin than against penicillin. Profound changes in the metabolism of the resistant organisms have been reported. Methods for determining metabolic processes in bacteria are crude and it is entirely possible that with less resistant organisms the changes in metabolism are not detectable. Resistance development to penicillin appears to be a reversion toward a more primitive saprophytic type of metabolism. It also may well be that streptomycin-resistant organisms tend toward saprophytism. It may seem far fetched to consider the necessity of streptomycin for growth of meningococci (213), *B. subtilis* (237), *S. aureus*, *E. coli*, *P. aeruginosa*, and *P. morgani* (238) as a tendency toward saprophytic existence, since Kushnick *et al.* (237) have only tested their resistant *B. subtilis* for growth in a synthetic medium. It is significant, however, in this respect, that only the resistant organism was able to synthesize its nitrogenous requirements from ammonium chloride. It is much to be regretted that in the light of the large amount of work being done on antibiotics relatively so little research is being directed towards the fundamentals of antibiotic reactions. This criticism may be unjust; the huge difficulties to be overcome in this type of research tend to preclude a plethora of publications.

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THE MODE OF ACTION OF CHEMOTHERAPEUTIC AGENTS

By Rollin D. Hotchkiss

The Rockefeller Institute for Medical Research, New York, N.Y.

For the purposes of the present review, chemotherapeutic agents are considered to include all defined preparations which have a pronounced effect upon the viability, or biological properties, of microorganisms, irrespective of whether or not these agents have achieved a recognized practical place in therapy. Naturally, sulfonamides, penicillin, and streptomycin dominate this field. However, antiseptics, toxins, and antibacterial agents of various sorts will be dealt with in this review, to the extent to which something can be said about the nature of their interaction with protoplasm. Mere observation of antimicrobial action, e.g., bacteriostasis, or bactericidal effect, is not necessarily recorded here, even when these data are available for an elaborate series of chemical modifications of the agent.

The period covered is principally from January, 1947 to March, 1948. Work of the preceding year or two which has interest in connection with current studies is sometimes referred to by the authors' names; a bibliographic reference is not generally given if the earlier work is referred to in the current publications.

Subjects not completely covered in this review are: agents toxic to viruses, parasites, or fungi; development of drug-resistant strains; behavior of chemotherapeutic agents in the animal host; relations between structure and activity; and general aspects of inhibitory analogues. Exact species and strains of microorganisms used will in the main be specified only when this feature seems to be a significant factor in the work under discussion. Recent reviews dealing with mechanisms of narcotic action (1), antibiotics (2), antimalarials (3, 4), and metabolite inhibition (5, 6) may be consulted for certain of these subjects.

General Concepts of Antibacterial Action

The phenomena of antimicrobial action are observed at many levels. An agent has generally first of all been observed to limit the growth, e.g., of bacteria. Frequently this action can be classified with some accuracy as either bacteriostatic or bactericidal (inhibi-

tory to growth or lethal). However, at this point the complexity of biological matter begins to make itself felt—it now appears that some agents may prevent or interfere with growth and, in so doing, initiate characteristic processes which lead to death of the organisms. Since these lethal processes apparently do not occur if the environment is unsuitable for growth, the action is neither bacteriostatic nor bactericidal in the simple sense of those terms.

Elucidation of the mechanism of antibacterial action is likely to require information at all of the following levels: (*a*) the physical-chemical process by which the agent attains a toxic concentration upon or within the affected cell (simple diffusion, adsorption, active absorption); (*b*) the interaction of agent at this concentration with some morphological element, or with some metabolic constituent, of the cell; (*c*) the interference with a normal cellular function occasioned by this interaction; and (*d*) the alteration, gradual or immediate, in the biochemical growth processes of the cell, by virtue of this interference. A full description of this last situation would obviously often be virtually a description of the total life processes of a cell. Needless to say, it has never been approached. Since biochemical processes of growth are largely unknown, the student of antibacterial mechanisms usually finds himself studying new aspects of normal metabolism or else limits himself to studying the effect of agents upon certain known processes included within the range of his knowledge or interest.

The four hypothetical stages of antimicrobial action, accumulation, interaction, interference, and alteration, should be kept in mind as we pass on to specific studies on the mode of action of chemotherapeutic agents. No study to be reported upon here approaches completion in this sense; it is not even possible in this review to discuss each topic from such a rigid standpoint. Nevertheless, the reader may be able to integrate each new fact or hypothesis in a mental framework based upon this somewhat comprehensive outlook, and if he thereby becomes aware of the areas of ignorance and surmise that surround the facts, so much the better. The mechanism of the sulfonamide inhibition, which is more completely pictured than any other, will be analyzed more or less in the sequence mentioned; in other cases it will be necessary to proceed in the reverse order since most of our observations concern alterations in treated cells.

We stress here this fourfold sequence of events because no anti-

microbial action is explained until something can be said upon all four of these aspects. The literature may contain a report indicating, for example, the interference of some antibacterial agent with a certain enzyme system, frequently a cell-free system isolated from some mammalian tissue or other diverse species of cell. It is necessary to question whether an effective concentration of agent could have been attained around or within the susceptible cell, at least at some local point. It is desirable to know whether an observed inhibition is due to an interaction with the enzyme, its substrate, or a coenzyme or cofactor. Finally, it is by no means to be assumed that the inhibition of this enzyme, if it does occur in the growth environment, will necessarily so alter the cell that it cannot grow. Many cases are known in which cells, through some change in the nutritional environment, have been observed to grow with an altered enzymatic composition (not genetically different) or with diminished formation of some product of enzyme action.

A great variety of experimental approaches in this field are more or less obvious straightforward attempts to observe one or more of the four events, accumulation, interaction, interference, and alteration. Before leaving the general aspects of the effects of drugs upon protoplasm, it would be well to mention that there are two frequently followed approaches, not quite so obvious or straightforward, which also are directed toward these same objectives. One is the empirical discovery and prominent use of substances counteracting the toxic drug; the other is the comparison of susceptible and resistant strains or species of cells. The former approach allows one to control, limit, or terminate the effects of the drug and also frequently gives indirect insight into the nature of its reactions with cellular constituents. The second approach is often pursued in the hope that observable differences between resistant and sensitive cells can be related to those functions with which the drug interferes. This hope is least valid when different species are compared but remains, at best, circumstantial evidence even when unrelated, accidentally encountered, strains of the same species are compared. There would seem to be greater possibility of drawing valid conclusions when the resistant and sensitive strains are related to each other by a more or less reproducible series of mutation and selection steps. Even here, we may conceive that a single mutation involving, for example, the ability to substitute chemically and detoxify a certain drug may also independently

lead to the chemical substitution of some metabolically unrelated normal constituent of the cell and produce as an observable result a change in metabolism, staining reaction, or morphology that is unconnected with the action of the drug.

SULFONAMIDES

Inhibition of pteroic acid synthesis.—It now appears that a major effect of sulfonamides as a group is to prevent the synthesis by bacteria of pteroyl compounds, derivatives of *p*-aminobenzoic acid which are related to folic acid. Those species, bacterial or otherwise (including mammalian hosts) which require a pteroyl compound as a growth factor and apparently therefore cannot synthesize it from *p*-aminobenzoic acid, are relatively insusceptible to the sulfonamides. This important development, already anticipated in recent *Annual Reviews*, is to a large extent the fruition of the "essential metabolite inhibition" hypothesis outlined by Woods & Fildes in 1940, as modified by the conception, especially due to McIlwain, that competitive inhibition is mainly effective when the metabolite inhibited is an essential growth factor supplied from outside and synthesized by the organism into a conjugated form. The recent significant work of Lampen & Jones (7) has furnished the basic pattern which now underlies all current concepts of sulfonamide action. Their findings, in turn, depended upon the brilliant work of a group of investigators at the Lederle Laboratories, who revealed that the *Lactobacillus casei* growth factor from liver, which may be considered identical with folic acid, is a derivative of *p*-aminobenzoic acid, namely, N-[4{[(2-amino-4-hydroxy-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid. The detailed publication of the chemical work has just appeared during the current year (8).

Certain lactic acid bacteria, such as *L. casei* and *Streptococcus faecalis* R, require for their growth this pteridyl-aminobenzoyl-glutamic acid, usually designated pteroylglutamic acid (PGA). Lampen & Jones (7) demonstrated that these organisms, growing in the presence of small amounts of PGA, were virtually unaffected by even high concentrations of sulfadiazine. This type of inhibition is described as noncompetitive inhibition, since a small amount of pteroyl compound will counteract equally well low or high concentrations of sulfonamide. With these species, which do not use *p*-aminobenzoic acid (PAB) for growth, sulfonamides and PAB, in

any combination, have virtually no effect upon growth. At the opposite extreme are those organisms, such as *Escherichia coli*, staphylococci, and pneumococci, which are highly sensitive to sulfonamides and presumably can use PAB for growth. In these species, as is now well known, PAB competitively inhibits sulfonamide antagonism of growth; PGA, however, does not (7), otherwise it is possible that folic acid in the animal body would counteract sulfonamide therapy. Petering, Delor & Murray (9) find PGA similarly ineffective toward a strain of *S. hemolyticus* (group B).

In between the extremes, sulfonamide-resistant *L. casei* and the sensitive *E. coli*, in their relations to PGA, are certain streptococci, *S. faecalis* (Ralston) and *S. zymogenes* (*26Cl*). These organisms are able to grow in the absence of PGA, but growth is nevertheless greatly stimulated by added PGA. While these organisms are being forced to synthesize their own pteroyl compounds, they are exquisitely sensitive to sulfonamides; but in an environment containing amounts of pteroyl compounds suitable for growth of a pteroyl-dependent organism, they grow and are unaffected by sulfonamides (7). *L. arabinosus* is also intermediate, and it is likewise resistant to sulfonamides when growing upon pteroyl derivatives (10). For this species, these latter derivatives are less effective than PAB itself for growth [see also (11)]; and available preparations of pteroyl compounds, although as a whole noncompetitively inhibiting sulfonamide, gave inconclusive results at minimal concentrations, since a small content of PAB (or other competitively inhibiting amine) was present. Morgan (12) has observed inhibition of the action of sulfadiazine upon psittacosis virus in infected chick embryos. The sulfonamide was inhibited competitively by PAB and apparently noncompetitively by PGA, much as in the bacteria just mentioned.

From this work, Lampen & Jones (10) concluded that sulfonamides, long thought to interfere with metabolism of PAB, are able to block specifically its conversion into pteroyl compounds. Whenever these compounds are provided preformed, those organisms able to use them, including those organisms normally dependent upon them for growth, are resistant to all ordinary concentrations of sulfonamides. Supporting this had been Miller's finding in 1944 that *E. coli* produced less folic acid when growing in the presence of low concentrations of sulfonamides.

In growth promotion, or sulfonamide inhibition, pteroic acid

and its conjugates with either one (PGA) or three residues of glutamic acid are, in general, effective for several species of lactic acid bacteria; a conjugate containing seven residues of glutamic acid is not. Pteroic acid is considerably less effective toward *L. casei* than toward the enterococci. Rhizopterin, identified chemically by Wolf, Anderson *et al.* of the Merck Research Laboratories (13) as N-formyl pteroic acid, is a growth factor effective for *S. lactis* R. (*S. faecalis* R), but inactive for *L. casei*. Presumably formyl pteroic acid, like PGA, circumvents the sulfonamide-sensitive synthetic step for this and doubtless for some related species. Martin, Tolman & Moss (14) report that pteroic acid, in contrast to PGA, does inhibit sulfathiazole for staphylococci. In view of the contrast, these authors are inclined to think that pteroic acid is a more important intermediate than PGA for this species. It does not appear reasonable to assume, alternatively, that PGA does not penetrate this cell, since it was stated in the same report to have an effect upon cells poisoned with a methyl-folic acid. On the other hand, the concentrations of pteroic acid used were relatively high, and no evidence is provided concerning (*a*) the noncompetitive nature of the inhibition, (*b*) the exclusion of the free PAB in the sample used, or (*c*) the possibility that the cultures were allowed enough time to "adapt" to pteroic acid utilization.[1] By and large, it does not yet appear that pteroic acid can be utilized as an equivalent to its conjugate, PGA.

The comparison of the activity of pteroic acid with that of PGA is of interest in connection with the analogous pair, PAB and *p*-aminobenzoylglutamic acid (PABG). Various workers, including those just quoted (7, 10, 11, 14), have failed to observe in a number of species a greater effectiveness of PABG than of PAB, reported by Auhagen for *Streptobacterium plantarum* some years ago. For *L. arabinosus* it has been suggested that the effective substance is PAB, liberated from PABG (10, 11) or, according to Sarett (11),

[1] The role of adaptation (which is usually the selection of an occasional mutant property) needs to be considered in any cases of apparent reversal of growth inhibition. The observation of normal growth rates displaying no unusual lag in relatively short periods of growth should be a minimal requirement, and it is advisable at times to compare the growth requirements of the recovered strain and the stock culture. It is obviously a different thing to say that "factor X is a growth factor for organism Y" and to say only that "a strain of organism Y can be recovered which will utilize growth factor X."

from the pteroyl conjugates as well. Lampen & Jones (10) suggest that the hydrolysis of PABG is inhibited by high concentrations of sulfapyridine; but in view of their own observation that the data suggest an adaptation of the organisms to use PABG, it seems possible that sulfapyridine merely decreases the amount of total growth so that the necessary adaptations or mutations do not have opportunity to occur.

Emerson (15) has obtained a *Neurospora* mutant, the growth of which requires sulfanilamide and is actually inhibited by small amounts of PAB. The need for sulfanilamide does not seem to be due to an abnormally high production of PAB by this mutant strain; indeed, a double mutant can be obtained which requires both sulfanilamide and PAB at a molar ratio of around one thousand, departures from this ratio leading to inhibition of growth. With both of these sulfanilamide-requiring strains, one sees the higher affinity for PAB characteristic of the inhibition phenomena in susceptible bacteria. Emerson points out that this could be one indication that for normal growth of these *Neurospora* both substances interact with the same enzyme system, just as they are commonly assumed to do in growth inhibition of PAB-requiring, sulfonamide-sensitive bacteria.

Concerning the actual physical interaction of sulfonamides with an enzyme building PAB up into pteroyl derivatives or their precursors there is little information. Earlier conclusions as to the role of electronic structure and spatial configuration have to be considered. Havinga & Veldstra (16, 17) find a general agreement in the ultraviolet absorption, electrometric titration, and polarographic behavior of PAB and the active, but not the inactive, sulfonamides. They attempt to calculate the collision frequency of drug and metabolite with cell receptors and make the interesting suggestion that the antagonist of a natural metabolite must be used in concentration high enough to block the receptors during most of the cell division cycle, while the metabolite may very likely be utilized immediately after a single collision. Erlenmeyer, Waldi & Sorkin (18) point to the parallels between the phenomenon of structural analogues inhibiting the syntheses utilizing a related metabolite and that of certain substances inhibiting the development of a particular crystal face in a structurally related substance. In neither case does the inhibitor seem to have much effect upon the end product after the building up process has al-

ready occurred. Tschesche (19) has suggested that the sulfonamides replace the PAB or PABG at a stage when they react with pteridinealdehyde. It might be recalled in this connection that sulfonamides substituted on the aromatic amino group are generally quite inactive toward bacteria.

Other substances recently reported to have strong bacteriostatic action inhibited by PAB are: p-aminosalicylic acid, acting on tubercle bacilli (20, 21) and p-aminobenzenephosphonous acid, tested upon *E. coli* (22).

Metabolic function of folic acid.—We now turn to the question of what processes in bacterial growth are critically altered as a result of failure to synthesize pteroyl compounds.

Various observers during the past several years had noted that thymine and certain purines tended to counteract, noncompetitively, sulfonamide inhibition of growth. For some time it has been suggested on this ground that PAB may be involved in the natural synthesis of purines and pyrimidines. The concentration of thymine or purine needed for effect is one thousand or more times that of pteroylglutamic acid (7, 10). This might mean that (*a*) thymine and purines were inefficient precursors of the pyrimidine moiety of pteroic acid derivatives, or that (*b*) pteroic acid derivatives form part of a catalytic system involved in the production of many times their weight of purines and thymine.

That the first possibility is not likely is indicated by several lines of observations. Several workers had shown before 1946 that thymine and purine could allow growth in folic acid-deficient media [recently also (23, 24, 25)]. Stokes (26) had found no evidence that organisms growing upon thymine and purine produced folic acid and had postulated that folic acid acted catalytically in the synthesis of thymine. Sarett (11) did not find any change in PGA synthesis from PAB occasioned by growing *L. arabinosus* in thymine.

Work with certain structural analogues of PGA has also indicated that pteroyl compounds function in purine and thymine synthesis. Hutchings, Mowat *et al.* (27) found that pteroylaspartic acid, which competes with PGA toward *L. casei* and *S. faecalis*, has no effect upon growth of these organisms in the presence of thymine. Here again, we conclude that the substances which inhibit a growth factor noncompetitively do so because they are end products of the normal action of that growth factor.

In this and other work to be described, the "inhibition index" (the molar ratio of drug to metabolite just giving inhibition) is used to some extent as an indicator of metabolic pathways. Shive & Roberts (28) had pointed out that this index[2] should have characteristic values depending upon which end product of the inhibited metabolite is the growth-limiting factor. By determining the index in different basal media we obtain a measure reflecting the relative importance of the various roles the inhibited system may have, even of a role for which the end product has not been isolated or identified. Knowledge of variations of the index in different localities in the animal body, and in different species, should help one to understand the efficacy or inefficacy of a drug in chemotherapy (28). Rogers & Shive (24), e.g., calculate the molar ratio of a crude methylfolic acid antagonist to folic acid just giving inhibition for *L. casei* in a purine-deficient medium as thirty, and thymine does not alter this. If adenine, guanine, hypoxanthine, or xanthine is added the ratio becomes one hundred, indicating that a smaller amount of PGA is now needed. Upon adding thymine, as well as a purine, the ration is raised to one thousand, indicating that a large part of the PGA requirement is satisfied by supplying thymine and a purine. Stokstad *et al.* (25), using the same folic acid derivative, report that it inhibits growth of *S. faecalis* upon all combinations of PGA, thymine, and adenine, except those in which both thymine and adenine are provided. This is compatible with the supposed role of PGA or related compounds in the synthesis of both thymine and one or more purines.

On the other hand, Hall (29) finds it unlikely that folic acid is a causative agent in the production of thymine, principally because his preparation of crude folic acid, added with thymine, stimulated acid production. Evidently PGA did not behave in the same way, and Hall's objection depends upon his insistence that folic acid represents more than a pteroyl compound. In any case, his results would very likely have been different if he had added both thymine and purine.

Hitchings, Elion & VanderWerff (23, 30) have continued their work with inhibitory substances related to the purines. These can inhibit growth occurring with PGA. An isomer of adenine, 2-

[2] Shive uses the ratio for complete inhibition as an index, while many others in the field prefer to use the more sharply defined 50 per cent inhibition ratio.

aminopurine, can inhibit growth and it is antagonized noncompetitively by thymine, and apparently competitively by adenine or PGA (30).

A number of structural analogues of pteroyl compounds have been made, partly on account of their possible effects upon neoplasms, which cannot be dealt with here. A benzimidazolyl- (31), and a quinazolyl- (32), derivative of *p*-aminobenzoylglutamic acid had weak folic acid activity, throwing some doubt on the absolute specificity of the pteridyl grouping in folic acid. Analogues which are inhibitory to bacterial growth include: (*a*) aspartic analogues already mentioned (27, 33), antagonized competitively by PGA or, less effectively, by pteroic acid; (*b*) a crude synthetic methylpteroic acid and methylpteroylglutamic acid (24, 25, 34), already discussed; (*c*) several methylpteroyl compounds related to these last, but reported to be 7-methyl derivatives (14, 33, 35), competitive inhibitors toward PGA; (*d*) analogues bearing an amino group substituted for the hydroxyl group in the pteridine nucleus (36, 37, 38); (*e*) sulfonyl analogues (31); and (*f*) analogues in which the pteridine rings are modified (31, 37, 38), or the whole structure replaced by simple quinoxaline (39). It is of interest that sulfathiazole at certain levels is said to counteract 7-methylfolic acid inhibition (14) and that certain amino analogues are counteracted by PGA only in presence of small amounts of sulfonamide (38).

Concerning the mechanism of purine synthesis, studies with isotopically labeled substrates, not covered here, are beginning to show that these molecules can be built up from such substances as carbon dioxide, ammonia, acetate, glycine, etc. The manner in which PGA participates is not known. However, Stetten & Fox observed some years ago that a cyclic nitrogen compound accumulated in *E. coli* cultures poisoned with sulfonamide. Shive *et al.* (40) have shown this compound to be a 5-amino-4-imidazolcarboxamide, a substance possessing the carbon and nitrogen skeleton of a purine, lacking only one carbon atom. They suggested that this substance was a natural precursor, normally transformed into purines by addition of one carbon atom through the intervention of an enzymatic system in which PGA plays a part. Ravel, Eakin & Shive (41) showed that glycine could be used in the synthesis of this imidazol. The observation, mentioned already, that the *S. faecalis* R factor, rhizopterin, was a N-formylpteroic acid (13) stimulated Gordon *et al.* (42) to prepare formylpteroylglutamic

acid. This was fully as effective as PGA in favoring growth, and it and formylpteroic acid were both more effective than PGA in counteracting methylfolic acid inhibition. Formylation of methylfolic acid greatly increased its inhibitory capacity. Such results suggest that the N-formyl group in these substances may have a function, and the authors offered the hypothesis that it may furnish the carbon atom for the completion of the purine structure from the imidazol precursor. It is, however, difficult to see how the formylation of one molecule of the "catalyst" can have an appreciable effect upon the rate of formation of many molecules of purine, if the catalyst loses its formyl group each time a purine molecule is produced.

That PGA may be involved in other functions than the supposed synthesis of purines and thymine is still suspected, especially since growth on the latter substances is frequently not as rapid or prompt as that upon PGA. Furthermore, Kalckar & Klenow (43) have observed that PGA inhibits strongly xanthopterin oxidase and xanthine oxidase, being slowly converted anaerobically by the former enzyme preparation into an altered form. Also, Totter, Sims & Day (44) reported that large amounts of PGA counteract the effect of cyanide, caffeine, or hydrogen peroxide, suggesting a possible relationship to porphyrin enzyme production.

Other effects of sulfonamides.—It has been mentioned that folic acid may have other functions than the synthesis of purines and thymine; there is some evidence that PAB, too, has other functions, which may or may not involve the pteroyl compounds. It has long been known that methionine was another substance which, under some conditions, could antagonize the effects of sulfonamides and presumably was synthesized in some way by a PAB-containing system. Shive & Roberts (28) showed that the sulfanilamide PAB inhibition index for a methionine-free medium indicated a relatively high dependence upon PAB. These authors confirmed the earlier work of Wyss, Rubin & Strandskov showing that 2-chloro-4-aminobenzoic acid was a PAB inhibitor specifically antagonized by methionine. Strandskov (45) reports that this inhibitor also differs from sulfonamides in the ease of development and the specificity of resistance toward it shown by bacteria.

Altman (46) found appreciable inhibition by five different sulfonamides of (yeast?) zwischenferment, partially competitive with

the coenzyme, triphosphopyridine nucleotide, but not reversed by PAB. A corresponding enzyme was demonstrated in *E. coli* but apparently not tested with sulfonamide; various other enzymes remained essentially unaffected by sulfonamides. Miller, Bruno & Berglund (47) found cells grown in sulfathiazole deficient not only in folic acid but also in biotin and pantothenic acid. It was the last factor which was most strikingly raised to normal in a sulfonamide-resistant strain; this type of resistance does not appear to be the same as that shown by *S. faecalis*, for example. Fisher & Armstrong (48, 49) find sulfonamides partially to inhibit bacterial respiration, but this is not unexpected, inasmuch as by adding the drug one is merely converting a suspension of growing cells into a suspension of "resting" cells. It might be mentioned that in their work respiratory rates are corrected for changing amount of bacterial mass, which was not done in a previous series of similar investigations by Sevag and associates. Youmans (50) reports that sulfanilamide affects immediately cells from the late phase of growth, while a characteristic lag period occurs before it affects cells from an exponentially increasing population. Gale (51) has stated that, after being grown in sulfathiazole, staphylococci have a diminished ability to couple glutamic acid into peptide or protein form.

Penicillin

At the present time there is little indication of differences in the mode of action of the various penicillins, F, G, K, X, etc., and in what follows the generic term penicillin is probably best construed as applying to all of the chemical species until more is known.

Physiological alterations occasioned by growing in penicillin.— For some time it has been realized that susceptible bacteria if attempting to grow in the presence of penicillin were rapidly rendered nonviable, whereas nonproliferating cultures remained viable and relatively unaffected. According to Yanagita & Suzuki (52) the gradual lysis of staphylococci by penicillin can be observed in dilute peptone and, without growth, in cysteine. Several workers recently have followed the rate of killing of staphylococci growing in the presence of penicillin. Parker & Marsh (53), by making successive plate counts, showed that viability begins to decrease after a lag period which is longer for lower concentrations of penicillin.

Organisms exposed to the agent for a time, then washed, and transferred into normal media continued to decrease in viability for a time depending upon the extent and duration of exposure. Schuler (54) measured oxygen uptake of proliferating cultures to obtain a continuous record of the effect of penicillin and streptomycin. He, too, found evidence of a lag dependent upon concentration and furthermore reported that the respiration eventually diminished to a steady level also dependent upon drug concentration. Hirsch & Dosdogru (55), also using the same technique, found similar results with both agents, except that with penicillin at low concentrations there was a pronounced immediate effect followed by lysis, while lysis did not supervene in higher concentrations. According to Eagle (56), the rate of killing is not increased beyond a certain maximal rate no matter how much penicillin is present. Besides staphylococci, other organisms showing essentially the same responses are streptococci, pneumococci, spirochetes (56), and *E. coli* (with much higher levels of penicillin) (52, 54).

Pratt & Dufrenoy have found that 4×10^{-6} *M* cobalt chloride enhanced the action of penicillin upon bacteria, especially upon staphyloccoci. The effect was demonstrable in cup assays *in vitro* (57) and in animal protection experiments toward *Eberthella typhosa* (58). This finding is of interest in connection with the conclusions of Albert, Rubbo *et al.* (59), discussed below (p. 208), that traces of cobalt appear to have a growth function for gram positive bacteria. Dufrenoy & Pratt (60, 61) also have performed color tests and precipitation reactions in the areas surrounding an inhibition zone on agar plates, finding evidence of various metabolic products in the region where visible growth occurs. These results and the findings with cobalt are discussed in terms of sulfhydryl groups and levels of oxidation.

It is known from previous work (62 to 66) that during growth inhibition in penicillin bacteria often produced pleomorphic diphtheroid, filamentous, or "swollen" forms. Klimek, Cavallito & Bailey (66) have found that repeated selection of penicillin-resistant staphylococci eventually gave a cocco-bacillary or diphtheroid, gram negative organism. Bellamy & Klimek (67) report that this resistant organism is a slow-growing obligate aerobe which can rather easily return to sensitivity, becoming again a gram positive coccus.

Metabolic alterations occasioned by growing in penicillin.—In

an important paper, Gale & Taylor (68) report that staphylococci and streptococci grown in low concentrations of penicillin have almost completely lost the ability to accumulate free glutamic acid inside the cell. On the other hand, further conversions of glutamic acid, the accumulation of lysine, and respiratory and glycolytic activity are normal in such cells. The decrease in glutamic acid accumulation is manifested by cells in media containing penicillin or by washed suspensions of bacteria that have been grown in penicillin, but washed normal cells appear to be unchanged in the presence of penicillin. There is thus some reason to think that a specific injury has been done to the cell during growth in the presence of penicillin, and it seems reasonable to suppose that the resultant inability to use glutamic acid may prevent growth. It is the process of accumulation in free form (Gale calls this "assimilation") of glutamic acid (or glutamine) that is affected; the further building up internally of glutamic acid compounds (51) is not. Lysine accumulation, which appears to be a process of physical diffusion, remains unaffected; other amino acids were not investigated.

Possibly connected with the effect mentioned is the finding of Schwartzman (69) that dicarboxylic and basic amino acids, and cystine, counteracted to some extent the effects of penicillin upon growing *E. coli*. Hotchkiss (70) had been studying the incorporation of various mixtures of amino acids into protein by respiring washed staphylococci, and on this system crystalline penicillin G had no effect (71). After the appearance of Gale & Taylor's preliminary report (72) the same worker tried staphylococci grown in penicillin but even then did not find a decrease in capacity to take up glutamic acid alone or in mixtures (71). In this work the total amino acid utilization was measured, and this is normally from ten to fifty times as great as any accumulation of free amino acid; but a decrease in uptake of glutamic acid or other individual should only thereby have appeared the greater. While Gale & Taylor used commercial penicillin with 33 to 50 per cent of the activity of penicillin G, it is probable that the failure of this limited attempt to confirm their interesting findings derives from differences in the organism or cultural conditions.

Subsequently Gale (73) has shown that staphylococci, selected for progressively increasing penicillin resistance, had less and less capacity to concentrate free glutamic acid. A still more far-reaching alteration of metabolism tending to link penicillin-

resistance with amino acid utilization has been noted by Gale & Rodwell (74). The more highly resistant derived staphylococcal strains [including those obtained by Klimek, Cavallito & Bailey (66)] are in their growth increasingly independent of external supply of the dozen or more amino acids required by the parent strains. The most highly resistant organisms are gram negative, grow without added amino acids, and do not accumulate any free glutamic acid internally. It appears as if the normal staphylococcus has compensated for a loss of synthetic abilities by learning to store a supply of free amino acid, incidentally becoming gram positive, and for a reason as yet not clear, penicillin-sensitive. More recently still, Gale (75) has found that staphylococci selected step by step for ability to dispense with amino acid growth requirements eventually became resistant to two thousand to five thousand times more penicillin without ever having been exposed to this agent. The last is a remarkable finding and rather convincingly indicates that dependence upon preformed amino acids in some way involves a penicillin-sensitive function.

Bellamy & Klimek (76) have added to the impression that penicillin-resistance involves a profound alteration of metabolism. They find that the highly resistant gram negative variant already mentioned is an aerobe, not producing acid from lactose, maltose, sucrose, mannitol, or galactose, not reducing nitrate, and independent of nicotinic acid source. All but the last of these characters suggest the relative loss of enzymatic abilities rather than a gain, such as is found with respect to amino acid synthesis.

It remains a mystery why cells must be exposed to penicillin in a growth medium before an inhibitory effect develops. It should be pointed out that exposure in this fashion (68) imposes in the main only the requirement that the cells develop a metabolic block, be it simple or complex, which will eventually limit growth. Development of penicillin resistance (66, 74) imposes this requirement and, in addition, the further one that some successful metabolic by-pass must be found in one or, as was demonstrated by Demerec, many separate mutational steps. It is to be hoped that the fundamental metabolic block occasioned by penicillin may eventually be demonstrated without the occurrence of growth.

Earlier work and certain current investigations (77, 78, 79) have indicated that penicillin-resistance does not necessarily involve production of penicillinase.

Price (80) and Krueger, Cohn & Noble (81) have recovered

(somewhat reduced) yields of bacteriophage from staphylococci treated with penicillin, although under their conditions no growth of the host bacteria could be observed turbidimetrically. Price (82) has further stated that penicillin has an effect similar to nicotinic acid, suggesting that penicillin prevents the host cell from using up this vitamin and in this way favors phage multiplication. There was no indication that penicillin had other than an indirect effect upon utilization of nicotinic acid, secondary to inhibition of bacterial growth. In this connection we may recall that resistant staphylococci synthesized their own nicotinic acid (76).

Inhibition of enzyme systems by penicillin.—Krampitz & Werkman (83) observed a slowly developing oxidation of endogenous or exogenous ribonucleate by washed staphylococci and cells of a few other species. Penicillin G in relatively high concentration completely prevented the accumulation of the oxidizable substrate (probably ribose) but had no effect upon the oxidation itself. The agent may block some stage in the dissimilation and possibly, therefore, also the synthesis of ribonucleic acid. It is accordingly interesting to learn that Massart, Peeters & van Houcke (84) find penicillin G in very similar concentrations to inhibit ribonuclease acting upon the ribonucleic acid of yeast cells. Adenosinetriphosphatase in *Clostridium sporogenes* is reported by Gros & Macheboeuf (85) to be considerably inhibited by penicillin. All of these workers used high concentrations of penicillin and of cells: from 200 to 2,000 units per ml. containing 2 to 20 mg. of cells. It is difficult to know whether these conditions are comparable to those in growth inhibition [for example, 0.05 units per ml. inhibiting growth of perhaps 0.000 005 mg. per ml. (53), or somewhat more, of cells].

While there have been countless instances of lack of effect of penicillin upon various bacterial enzymes, toxins, etc., a few might be mentioned for their relevance here. This agent did not appreciably affect a number of oxidative systems (68, 83), amino acid utilization (68, 71), glycerophosphatase (85), nor muscle adenosinetriphosphatase (86). According to Torda & Wolff (87), however, penicillin in moderately large amounts had an inhibitory effect on acetylcholine production in a brain mince.

Evidences of penicillin combination or accumulation within the cell.—There is considerable evidence in the literature that sulfhydryl compounds can inactivate penicillin, and the suggestion has

been made by Cavallito & Bailey that penicillin can by virtue of this interaction inactivate biologically important sulfhydryl-containing enzymes. It is known that penicillin action is not reversed by cysteine (88), although this sulfhydryl compound added beforehand would fairly rapidly have destroyed the agent. Leonard (89) has recently found sodium thiosulfate and metabisulfite ineffective in destroying penicillin. This author points out that there is as yet no evidence that penicillin reacts with protein or enzyme sulfhydryl groups. Cavallito (90) is in agreement with this and calls attention to the possibility that particular spatial relations between the sulfhydryl and neighboring groups may give rise to considerable specificity. Fisher (91) points to a certain formal resemblance between the structures of penicillin and of glutathione.

As already mentioned, various difunctional amino acids antagonized to some degree the action of penicillin upon growing *E. coli* (69); methionine, threonine, and serine enhanced it. According to Pandalai & George (92), nucleic acid has a moderate antagonistic effect, even reversing the action of penicillin on staphylococci and other organisms to some extent. Nucleic acid was not one of the materials having appreciable effect on penicillin in the systematic investigation of Frieden & Frazier (93).

The observation that it is only growing cells which are notably affected by penicillin calls to mind the possibility that a metabolic process involved in growth may cause the accumulation or incorporation of penicillin into toxic concentration or combination at some point in the cell. The lag before sterilization begins, dependent as it is on concentration, seemed to Parker & Marsh (53) as conceivably a time in which either penicillin was accumulated or else a competing metabolite became exhausted. Synergism between penicillin and various other agents has frequently been reported. An interesting case was shown by Kirby & Dubos (94), viz., that growth of *Mycobacterium tuberculosis* is markedly inhibited when the wetting agent Tween 80 is present. The presence of this wetting agent, a mixture of polyoxyethylene derivatives of sorbitan oleates, rendered the otherwise virtually insensitive organism highly susceptible to penicillin. This might be construed as evidence that the vulnerable sites of the organism were not accessible to the inhibitor until a "wetting" or "penetrating" agent was added. Lysis, although indicated, was minimal and not pro-

gressive. Another factor which in certain media, and *in vivo*, determines accessibility of the various penicillins to bacteria is the extent of binding of these agents by serum albumin (95).

Streptomycin

Alteration of cells grown in streptomycin.—As already mentioned, streptomycin, like penicillin, has been observed (54, 55) to inhibit respiration of growing bacteria after a lag period depending upon concentration. Further evidence that it, like penicillin, is bactericidal principally for growing cells is furnished by Middlebrook & Yegian (96) working with tubercle bacilli, and by Hamre, Rake & Donovick (97) using *Klebsiella pneumoniae*. The latter workers found that thirty to fifty times greater concentrations of streptomycin were lethal to washed organisms although the survivors of this type of treatment were not inheritably more resistant. A similar observation was made by Strauss (98) with *E. coli*. Strauss also recovered large filamentous forms of various gram negative bacteria growing in streptomycin, similar to forms also seen by Welch, Price & Randall (99) in *Eberthella typhosa*.

Miller & Bohnhoff (100), by selecting for resistance, have repeatedly obtained an interesting meningococcal variant which requires streptomycin for growth *in vitro* or *in vivo*. Fermentation or immunological reactions were not notably different from those of parent strains.

A number of substances have been reported to antagonize streptomycin inhibition of growth, but the fragmentary and varied data do not suggest clearly defined metabolic alterations caused by the agent. Observations include antagonism by methionine, cystine, tyrosine, and peptones (101), by pyruvate, fumarate, and dicarboxylic acids (102), and by purines, pyrimidines, and urea (103) toward inhibition of *E. coli*. Various salts markedly interfere with the growth-preventing action of streptomycin on several bacterial species according to Green & Waksman (104) and are largely responsible for the effects of different peptone preparations. An antagonist first seen in brain tissue and plant peptones (105) was later stated by Rhymer *et al.* (106) to be lipositol. The presence of sugar residues in both inhibitor and antagonist was conceived as a possible basis for their competition.

Cells of *E. coli*, not propagated, but incubated (with a moderate amount of proliferation), in fumarate, succinate, glucose, or

glycerol, were affected if streptomycin was present during the incubation. Geiger (107) has shown that the drug prevented the large increase in amino acid oxidizing activity normally shown by the incubated suspensions, although it had no effect upon the oxidation itself if added after the incubation.

Effects of streptomycin upon enzyme systems of cells.—Streptomycin, rather unlike penicillin and the sulfonamides, appears to modify certain oxidation systems in washed bacteria. According to Fitzgerald & Bernheim (108), nonpathogenic mycobacteria, if they are able to oxidize benzoic acid, have this oxidation markedly depressed by approximately 1 μg. streptomycin per ml. Pathogenic strains, which do not oxidize benzoic acid although their endogenous respiration is stimulated by it, are unaffected. Since the effect of streptomycin upon growth is equivalent for pathogenic and nonpathogenic strains, the authors do not maintain that the inhibition of benzoic acid oxidation is involved in growth inhibition. Nevertheless, as resistance is developed toward the growth inhibiting effect of streptomycin this oxidizing function is progressively less affected.

Four recent notes detail other effects upon washed bacteria. Benham (109) reports that streptomycin, 300 units per mg. of *Eberthella typhosa*, stimulated endogenous respiration and increased the degree of oxidative utilization of glucose and other substrates. Henry *et al.* (110) find lower concentrations to inhibit oxidation of glycerol or lactate, etc., by *Bacillus cereus* and *Shigella dysenteriae*, resistant strains being unaffected. Wight & Burk (111) find little effect upon oxidation of amino acids, or decarboxylation, but anaerobic deamination of amino acids by *E. coli* is partly inhibited [see (107)]. In an early report of their work on the oxidation of endogenous and exogenous ribonucleate [see (83)] Krampitz, Green & Werkman (112) mention that streptomycin, like penicillin, had an inhibitory effect.

Interaction of streptomycin with cell constituents.—Cohen (113) found that streptomycin reacted as a polyvalent base, precipitating with nucleic acids in certain proportions. Massart, Peeters & van Houcke (114) find that it can displace acridine dyes from combination with the nucleic acid of living or dead yeast cells. In both cases the investigators are inclined to attribute biological significance to this reaction with nucleic acid.

Reagents combining with aldehyde groups (semicarbazide,

hydroxylamine) and oxidants, which convert them to a carboxyl group, were known to inactivate streptomycin, Nevertheless, it does not appear probable that this agent combines with some bacterial receptor by virtue of an aldehyde group, inasmuch as dihydrostreptomycin, which lacks this group, has similar activity (115, 116, 117). It has been suggested (115, 118) that reoxidation to streptomycin is carried out by the growing bacteria. Gray & Birkeland (117) find that the effect of the aldehyde group upon dehydrogenases is unrelated to streptomycin-sensitivity. Sulfhydryl destruction of streptomycin (118) does not seem probable in a biological system (119) and will not reverse its action (88).

Agents Inhibiting the Interactions of Viruses with Cells

An interesting recent development has centered around the biology and physiology of virus-cell interactions. In 1941, Hirst had shown that red cells from certain species are agglutinated by influenza virus and that the virus is bound to the cells. With incubation, however, a process which has some resemblance to an enzymatic one occurs, and virus is liberated virtually completely, leaving behind an altered, no longer agglutinable cell. Virus heated at 55°C. agglutinates but does not elute from red cells (120, 121). Ferret lung cells had also been shown to absorb and subsequently release the virus. Burnet, Horsfall, and their co-workers have since observed similar reactions of mumps, Newcastle disease, pneumonitis and other virus preparations with red cells, chick embryo, and lung tissue. Cholera vibrios, pneumococci, and sera have also yielded preparations apparently able enzymatically to destroy the virus receptors of normal red cells (122, 123,).

The conception that the viruses, in altering the red cells, have an enzymatic action upon a receptor present in or upon the cells suggests that soluble substrates for the virus enzyme might be extracted from susceptible red cells or other tissues. If the soluble substrate were as slowly acted upon as are cells, then it should evince an inhibitory effect upon the virus-cell reaction and conceivably might have a "chemotherapeutic" action in virus infection. Some fragments of this hopeful picture are beginning to appear in recent work and, at the same time, certain associated phenomena are being discovered.

In the earlier work Hirst had noted that normal sera inhibited virus hemagglutination. Friedewald, Miller & Whatley (124) re-

covered inhibitory extracts from various human tissues and serum. These workers were able to prepare inhibitor from red cells of those species showing virus hemagglutination, but not from non-agglutinable cells, including virus-treated chicken erythrocytes. The inhibition was partly reversed after a six-hour incubation, suggesting a release of virus from a virus-inhibitor combination. Bovarnick and associates found that lipid extracts of red cells which inhibited hemagglutination (125) became modified in further purification, giving rise to a chloroform-insoluble inhibitor which appeared to be, in part at least, a hetero-disperse polysaccharide (126). Some data was presented indicating that the inhibiting property is destroyed on incubation with virus. Serum inhibitor is also stated by Burnet, McCrea & Anderson (122) to be inactivated by influenza virus. Burnet (127) has reported that blood group O substance and a crude mucin inhibit influenza virus hemagglutination and are reduced in activity by incubation with the virus.

The relation of these inhibitory materials to the virus receptors is, however, problematical. Hirst (128) has recently furnished evidence that the red cell influenza virus receptor and the hemagglutination inhibitor from rabbit serum have several properties in common. Both are stable at elevated temperatures and at pH 10; both are inactivated by virus, trypsin, and periodate. The destruction by periodate is of unique interest since it was demonstrated that iodate and other oxidants were without effect. It was this finding of Hirst's, made public in 1946 (129), which originally suggested to him, to Burnet and to Green & Woolley that polysaccharides, which are vulnerable to periodate oxidation, might be involved in the reaction. The serum inhibitor can be visualized as a mucoprotein (128).

Indeed, a number of polysaccharides from various sources have been found to affect virus-cell interactions and in some cases even have a protective effect in virus infection. Certain pectins and gums were found by Green & Woolley (130) to inhibit agglutination of red cells by influenza virus, and it was noted that at higher concentrations these substances themselves had a hemagglutinating effect. These preparations also inhibited multiplication of virus in the chick embryo. Horsfall & McCarty (131) demonstrated that certain polysaccharides of bacterial origin interfered with pneumonitis virus multiplication in the mouse lung, pro-

tecting the animal from infection. The same materials did not appear to interfere with the virus-hemagglutination. Later, Ginsberg, Goebel & Horsfall (132, 133) found that the capsular polysaccharide of *Klebsiella pneumoniae*, Type B, was adsorbed to red cells, and even after washing, the cells would not react with mumps virus. The same polysaccharide prevented multiplication of this virus in the chick embryo, but did not affect virus-host cell combination. At the present time it appears that the polysaccharides in the investigations just mentioned have the capacity to react with the cells, and there is little if any evidence that they interact with the viruses. Virtually all of the effective polysaccharides are polyuronides, although not all of the polyuronides are effective; furthermore, the results differ with the virus used.

Tryptophane was shown by Anderson a few years ago to be necessary for the absorption of certain bacteriophages upon the host *E. coli*. An analogue of tryptophane, the 5-methyl derivative, did not inhibit absorption, but according to Cohen & Anderson is a potent growth inhibitor for phage and host. Cohen & Fowler (134) find that the multiplication of phage can be stopped at almost any stage of the latent period, and upon addition of tryptophane, the natural metabolite, multiplication is resumed.

Surface Active Antiseptics and Phenols

Another class of substances with pronounced effect upon microorganisms is the surface active agents, including soaps, synthetic detergents, wetting agents, etc. At high concentrations these are bactericidal and in smaller amounts they are often bacteriostatic; other microorganisms and tissues are also affected. Baker, Harrison & Miller a few years ago had indicated that these agents inhibited bacterial respiratory enzymes, but the correlation of this effect with antiseptic action was indirect and incomplete. Kuhn & Bielig had postulated that the effects upon cells were largely due to the denaturation of proteins. Valko & Du Bois (135) maintained that they were due to the blocking of oppositely charged groups in the cell. Hotchkiss (136) reported a close correlation of bactericidal effect with a cytolytic effect—the loss of cell solutes into the suspending medium. Inorganic phosphate, labile and stable phosphate esters, nitrogen compounds, amino acids, and pentose are liberated from gram positive bacteria and yeast under those conditions of concentration and time of exposure which

result in bacterial death. Cationic and anionic, highly and weakly ionized, surface active agents of diverse structures, including fatty acids, alkyl sulfates, and quaternary ammonium salts were effective. Even ordinary phenol and the alkylated and halogenated phenols had this effect at the killing concentration (137), which is rather high. Probably there is some concentration of every ionizing surface active agent at which it will kill and cytolyze the susceptible bacteria. The bacterial polypeptide tyrocidine, with which the investigation had begun, belongs to the cationic surface active class, as does the related "gramicidin S" (Soviet gramicidin), in contrast to the neutral gramicidin proper (71). In an elegant way, Gale & Taylor (138) confirmed the cytolytic effect by measuring the liberation of the individual amino acids, glutamic acid, arginine and lysine, and independently arrived at the conclusion that phenol acted like the typical surface active agents. Consistent with this observed cytolytic mechanism is the evidence of electron photomicrographs presented by Mitchell & Crowe (139) indicating alterations in the boundaries of cells from tyrocidine-lysed cultures. Johnson (140) had published similar pictures of tyrothricin-treated cells several years earlier without knowing of the analytical evidence of cytolysis.

It has not been demonstrated to what extent enzyme inhibition may be responsible for, or secondary to, a cytolytic effect upon cell membranes. However, the detergents can almost instantaneously cytolyze cells at 0°C., at which temperature no respiratory or hydrolytic enzyme activity is appreciable. Such cytolyzed cells are no longer viable, but they do not undergo bacteriolysis, actual clearing, unless they contain active autolytic enzymes, and the temperature is raised so that enzymatic action may go on. Moreover, the loss of intracellular coenzymes and solutes seems to be an adequate explanation for a virtually complete "inhibition" of respiratory functions and loss of ability to grow in subculture. The suggestion was made (137) that if the enzymes are not irreversibly damaged cytolyzed cells might regain metabolic or growth capacity in a medium containing an adequate concentration of the essential coenzymes and solutes. Substances not needed for normal growth might be required by the damaged cells. In this connection it is interesting to learn that McCulloch (141) has found milk to allow growth of cells unable to grow in ordinary broth after treatment with a cationic detergent.

In general, however, reversal of bactericidal action by surface active agents has not been effective, although certain phosphatides were shown by Baker, Harrison & Miller to protect cells if added first. Valko & Du Bois (135, 142) had reported such reversal by an oppositely charged detergent but had failed to demonstrate that more than an infinitesimal number of cells had survived (137), Later, Du Bois & Dibblee (143) and McCulloch (141) observed that survivors were far fewer than suspected from all-or-none tests, even following exposures well below the absolute killing level. Klein & Kardon (144) found no evidence of reversibility when quantitative criteria were applied, and even neutralization was ineffective toward gram positive bacteria. Roberts & Rahn (145) had used respiratory activity as a measure and were unable to reverse the toxic effect of cationic detergents and phenol. In a similar study, Weissman & Graf (146) were able to prevent, but not reverse, the effect of a quaternary ammonium soap on respiration by adding lecithin.

The bacteriostatic action of surface active agents, on the other hand, is by definition reversed when the agent is removed; therefore, it probably does not result from physical damage to the cell and may very possibly be linked to the inhibition of one or more specific enzymes, although not necessarily of the whole respiration. Various workers had expressed such a view.

The cytolytic effect of the surface active agents must depend upon an interaction with cell receptors. It had been suggested by Albert and also by Valko (142) that the first combination is with oppositely charged groups on or within the cell. Dyar (147) believes to have demonstrated, through staining, the absorption of a cationic agent by fixed bacteria and yeasts. According to Beck & Meier (148) yeast cells, like charcoal, maintain an adsorption equilibrium between free and bound agent. We may suppose that when the amount of bound surface active agent reaches a certain level processes occur similar to those produced by the same type of agents in hemolysis, investigated by Schulman and others.

Dyes, Quinones, and Related Compounds

The bacteriostatic action of the acridines is attributed by Albert, Rubbo *et al.* (149) to the competition between the dye cation and hydrogen ions or other cations for sites in the bacteria. The activity increases with the degree of cationic dissociation,

according to Rubbo (150), somewhat more rapidly than does the animal toxicity. Massart and co-workers (151, 152) find that hydrogen, sodium, magnesium, or aluminum ions counteract the following effects of acridines on yeast: (*a*) inhibition of respiration by high concentrations; (*b*) staining of nucleic acid; and (*c*) inhibition of growth by lower concentrations (less effectively counteracted). Silverman (153) reports that magnesium and calcium can also remove the growth inhibitory effect upon *E. coli* due to atabrine, which is a complex acridine derivative. The effects of atabrine upon metabolism of malaria parasites and bacteria, described earlier by Silverman and Evans, may perhaps be attributed to metal displacement.

Triphenylmethane dyes inhibit the intracellular conversion of glutamic acid to peptides and other products, as found by Gale & Mitchell (154) in washed gram positive bacteria. The inhibitory efficacy, the bacteriostatic activity, and the lipoid solubility all tended to increase together with increasing alkyl substitution in the triphenylmethane series. This finding lends interest to the reports by Treffers (155) and George & Pandalai (156) that penicillin and gentian violet together were considerably more inhibitory than either alone toward certain bacteria. This synergism seems reasonable in view of the previously mentioned ability of penicillin to render the growing cell less capable of taking up free glutamic acid, and the effect of the dyes to interfere with utilization of that which is taken up.

A number of antibacterial compounds, such as clavacin, citrinin, penicillic acid, and some synthetic unsaturated ketones, have a structural relationship to the quinones. These compounds are highly reactive, reacting among other things with sulfhydryl compounds. Geiger (157) has stated that the antibacterial, antifungal, unsaturated ketones inhibit sulfhydryl enzymes such as urease and some of the dehydrogenases. Hoffman-Ostenhof (158), however, reports that the effects of various quinones upon such enzymes is by no means correlated with their antibacterial efficacy and suggests that reactivity of the quinones with amino groups, or with oxidation-reduction systems, may be equally important. Citrinin, curiously enough, appears to give rise to sensitive, rather than resistant, variants of bacteria grown in its presence (159).

According to Ball, Anfinsen & Cooper (160), 2-hydroxy-3-alkylnaphthoquinines probably inhibit malaria parasite and yeast

respiration through an effect at the level between cytochromes *b* and *c*. The inhibition did not seem to be due to effects upon sulfhydryl systems generally, nor was it counteracted by cysteine. In luminous bacteria methylnaphthoquinone may, according to McElroy & Kipnis (161), inhibit luciferin oxidation, which itself is possibly the oxidation of a hydroquinone by a cytochrome system. Bueding, Peters & Waite (162) found the same quinone to inhibit aerobic glycolysis more than respiration in schistosomes. Yeast carboxylase activity is reduced by high concentrations of various quinones, ketones, and phenols (163).

Some part of the beneficial effects of salicylic acid in rheumatic fever may possibly be reaching explanation. Guerra (164) found that salicylate inhibited the spreading action of hyaluronidase in rabbit or human skin. However, neither Guerra nor Pike (165) was able to demonstrate an inhibition by salicylate *in vitro*, although Dorfman, Reimers & Ott (166) stated that higher concentrations do have this effect. Lowenthal & Gagnon (167), however, report that the quinone obtained by oxidation of gentisic acid, a natural metabolite of salicylic acid, is strongly inhibitory toward hyaluronidase. Gentisic acid, gentisuric acid, and other hydroquinones and quinones are highly inhibitory to the enzyme according to Meyer & Ragan (168). At the present time, therefore, it is possible to conceive that salicylate may function in part by inhibiting the action in tissue of the streptococcal hyaluronidase, or spreading factor.

The cyanine dyes, not so closely related to the quinones, also have yielded some active growth inhibitors. Brooker & Sweet (169) found bacteriostatic activity *in vitro* somewhat reversed by vitamins and crude tissue extracts, but no appreciable activity *in vivo* nor against various isolated enzyme systems. Welch *et al.* (170) found cyanines to be antifilarial; both *in vitro* and in the animal, adult filaria were killed and respiration was partly replaced by aerobic glycolysis with reduced glycogen synthesis.

Miscellaneous Antimicrobial Agents

Substances related to 8-hydroxyquinoline can form chelate complexes with various metal ions. Albert and co-workers (59) finds that the bacteriostatic action of these agents can be reversed by adding the suitable metal ions. Cobalt is effective for gram positive bacteria and the gram negative cocci. Inhibition of the

gram negative bacteria is reversed variously by iron, zinc, or copper.

It was reported (136) that dinitrophenol and sodium azide, shown by Clifton to prevent assimilation, and the antibacterial peptide gramicidin were all able to block the taking up of inorganic phosphate by respiring cells. Spiegelman & Kamen (171) stated that in yeast the first two agents prevented the exchange of phosphate from the nucleoprotein fraction, which, it was later shown, was primarily the exchange of an insoluble form of metaphosphate (172). However, Reiner & Spiegelman (173) found that dinitrophenol stimulated the fermentation of stored assimilated glucose, while azide did not have this effect. Azide was found by Stannard & Horecker (174) to be a potent inhibitor for a heart cytochrome oxidase preparation. These authors felt that the pH sensitivity of this cell-free system could not be ascribed to permeability; it should be remembered, however, that the cytochrome oxidase of tissue is located in intracellular particles (175).

Puck (176) states that the bactericidal effect of glycol "aerosols" is actually due to the rapid condensation of a lethal film of glycol and water upon the cells.

Novelli & Lipmann (177) report that phenylpantothenone, an analogue of pantothenic acid, suppressed the synthesis by yeast of coenzyme A, a pantothenic acid derivative (178), but had no effect upon the acetate-oxidizing activities of the coenzyme. According to Shive *et al.* (179) the synthesis of pantothenic acid itself in *E. coli* is prevented by cysteic acid, which inhibits competitively aspartic acid conversion into β-alanine. The growth inhibition is counteracted by dicarboxylic acids of the citric acid cycle, interpreted as an evidence that these are a product of pantothenic acid catalysis, via the condensation of oxaloacetate with pyruvate, which apparently cannot occur in the pantothenic-impoverished bacteria.

Fluoroacetate, closely resembling acetate in structure and size is found by Kalnitsky & Barron (180) to inhibit acetate oxidation in yeast. The inhibition is competitive but not altogether reversible; metabolism of other organic acids in *Corynebacteria* is inhibited.

Carboxylmethoxylamine is bacteriostatic at moderate concentrations (181) presumably by virtue of interaction with carbonyl compounds, which combine with it, and in sufficient amount counteract it.

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INHERITANCE OF IMMUNITY IN ANIMALS[1]

By John W. Gowen

Department of Genetics
Iowa State College, Ames, Iowa

Reactions characteristic of individuals within a species exposed to pathogens may vary from those causing no effect to those causing death. All grades of effects between these extremes are observed. Morbidity of various degrees is ordinarily the most frequent result. Unaffected individuals could be considered immunes, those which show morbidity to different degrees, resistants, and those which die, susceptibles.

Inheritance affects these reactions according to the processes necessary to bring about the disease. The normal organism is the resultant of the action of some thousands of genes (five thousand to thirty thousand, with possibly eight thousand as the likely number in *Drosophila*) [Gowen & Gay (1)]. The morbid condition may be due to a gene or group of genes substituted for the normal genes, their alleles, working on a substrate of tissue formed by the remaining normal genes. In this exchange the substituted gene is in truth the pathogen as it parasitizes the normal development of the host and leads to its death or unfitness for survival as contrasted with a host having the normal alleles. The severity of the effects of these lethal or semilethal genes depends upon the conditions surrounding the host. A favorable environment may counteract much of the bad inheritance; one that is poor may accentuate the defect. In a word, one unfavorable gene may destroy the individual that it and its associated normal genes had a part in beginning. Besides the host genes, the morbid condition may need the intervention of another factor, a bacterium, protozoa, or virus acting on the host to bring about the disease. Such pathological syndromes rest on three legs: the host gene complex, the pathogen gene complex, and the environmental complex. Vary any one of these complexes, and, depending upon the nature of the disease, a more or less extreme variation in the morbid condition will result [Gowen (2)]. Interest in the problem lies in understanding the in-

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teractions of these variables so that prevention and control of the morbid process may be accomplished.

Diseases Attributable to Host Genes

An almost unlimited variety of pathological conditions is the result of one to a limited number of genes among the otherwise normal complex found in the fertilized egg. Any one or group of such genes, which it is well to recall are always the alleles of normal genes, guides development of the egg to a type, a phenotype, which is so abnormal as to lead to its death or serious disability. The phenotypes resulting from the same gene or gene group rather uniformly express the same pathological syndrome. Different pathological types are the resultants of different abnormal gene groups, generally considered as semilethal or lethal genes depending on whether disability of the adult occurs or death at an early stage, i.e., before birth in mammals, takes place. These results are still cited and spread through the literature as though they were the only effects of inheritance on disease [Bauer (3)]. They are responsible for the misconception that a diagnosis of "hereditary" in the etiology of a disease carries the connotation "hopeless," in that nothing can be done for the cure of the condition. Genes causing pathological conditions are so numerous that only a representative sample of the carefully analyzed cases in animals is cited in Table I. Reviews presenting extensive summaries of these studies are found in Mohr (46), Gowen (2, 47), Perla & Marmorston (48), Lerner (49), Grüneberg (50), and others. For summaries on the human being, Baur, Fischer & Lenz (51), Blacker *et al.* (52), Macklin (53), and Gates (54) may be consulted.

The cases cited in Table I are chosen as representative of pathological conditions caused by single genes. The incompleteness of this sample is evidenced by the writer's more complete list of 201 lethal and semilethal genes causing other similar pathological conditions in these same species. In the human being, where more attention has been given to this type of inheritance than in any other species, Macklin (53) cites thirty-five hundred references and 210 lethal or semilethal conditions attributable to simple inheritance. Almost every organ, morphological or physiological process may be affected by one or more of these genes. Yet the number of such genes which have been isolated and studied are but few compared with the total number. In *Drosophila*, the form

TABLE I

DISEASE CONDITION IN WHICH LETHAL OR SEMILETHAL GENES ARE THE ETIOLOGICAL AGENT

Condition	Inheritance†	Animal	Investigator
Waltzing—nervous defect	r	Guinea pig	Ibsen (4)
Hydrocephalus	r	Mouse	Clark (5)
Absence of corpus callosum	r	Mouse	Keeler (6)
Dwarfism	r	Mouse	Snell (7)
Recessive anemia	r	Mouse	De Aberle (8) and Gowen & Gay (9)
Grey lethal	r	Mouse	Grüneberg (10)
Yellow lethal	r	Mouse	Cuénot (11)
Cataract (bilateral)	D	Rat	Smith & Barrantine (12)
Dwarfism	r	Rat	Lambert & Sciuchetti (13)
Cartilage and thorax effects	r	Rat	Grüneberg (14)
Ataxia—nervous disorder	r	Rabbit	Sawin *et al.* (15)
Hydrophthalmus (juvenile glaucoma)	r	Rabbit	Nachtsheim (16)
Pig jaw—shortened mandible	r	Dog	Phillips (17)
Leg achondroplasia	D	Dog	Stockard (18)
High uric acid excretion	r	Dog	Trimble & Keeler (19)
Hairless	r	Cat	Letard (20)
Hemophilia-like condition	r	Pig	Bogart & Muhrer (21)
Porphyrinuria	r	Pig	Clare & Stephens (22)
Catlin mark—Parted parietals	r	Pig	Hughes & Hart (23)
Split ears—cleft palate	D	Pig	Annett (24)
Amputated legs	r	Pig	Johnson (25)
Hairlessness	r	Pig	Roberts & Carroll (26)
Lethal grey—homozygous die	r	Sheep	Contescu & Epureanu (27)
Muscle contracture	r	Sheep	Roberts (28)
Paralysis—hind legs	r	Sheep	Rasmussen (29)
Muscle contracture	r	Cattle	Mohr (30)
Short spine	r	Cattle	Mohr & Wriedt (31)
Impacted molars	r	Cattle	Heizer & Hervey (32)
Epilepsy	D	Cattle	Atkeson *et al.* (33)
Congenital eye defect	r	Cattle	Detlefson & Yapp (34)
Porphyrinuria	r	Cattle	Fourie (35)
Cataract	r	Cattle	Gregory *et al.* (36)
Achondroplasia	r	Cattle	Mohr & Wriedt (37)
Epitheliogenesis imperfecta	r	Cattle	Hadley & Cole (38)
Atresia coli	r	Horse	Yamane (39)
Creeper—reduced extremities	D	Chicken	Landauer & Dunn (40)
Crooked neck dwarf	r	Chicken	Asmundson (41)
Short beak and long bones	r	Chicken	Landauer (42)
Chondrodystrophy	r	Chicken	Lamoreux (43)
Achondroplasia	r	Pigeon	Hollander (44)
Melanoma	*	Fish	Gordon (45)

* Interaction of D with A+B factors.

† D indicates dominant; r indicates recessive.

about which most is known, pathological changes attributable to lethal inheritance already number several thousand. In fact, it seems that nearly every normal gene in the fly is capable of change to an allele or a loss which will cause pathological types, identifiable through some change in the morphologic, physiologic, or embryologic development, and leading to death or disability at some stage in the development. The frequent and striking effects of this class of genes accounts for some writers considering them all that heredity has to contribute to medicine, whereas they constitute but a small part of what genetics means to pathology. An important consideration may be attached to these cases which is not true of the other genetic effects. For them a true immune type may be said to exist. The individual with proper genetic constitution, in general, will not show the defect, the immunity being conferred by the normal alleles of the pathological genes. But it is possible, by applying quite abnormal stimuli, such as high temperature in *Drosophila*, to cause the developing embryos to simulate pathological types which are due to gene mutations [Goldschmidt (55)]. The gene complex, genotype, is normal, but its action in guiding the developing embryo is altered by the stimulus, resulting in the abnormal phenotype. Even normal genic development, if properly stimulated, yields graded resistance rather than immunity.

The pandemic significance of these genes is expressed, as with infectious diseases, both horizontally and vertically in time. Atresia coli, caused by a lethal gene in horses, was spread over Japan after its introduction from Ohio, U.S.A. [Yamane (56)]. Hairless Jersey calves, unknown to the respective Texas and California owners, had common origin [Cook (57)]. In vertical time, the unbalanced gait of mice, waltzing, can be traced to 80 B.C., and dominant spotting with recessive lethal effect, to 1100 B.C. [Keeler (58)]. For man similar pathological phenotypes can be traced from the time of the Romans to the present day.

Genic and Environmental Interaction in Disease

Specific environmental stimuli interacting with particular genes may lead to particular disease syndromes. Genetically differentiated inbred lines of rats fed the same vitamin D-deficient diet showed differences in survival from 131 to 345 days for males and 132 to 267 days for females. Hereditary differences accounted

for somewhat more than half the variation [Gowen (59)]. Similar differences have been noted in vitamin B lack [Lamoreux & Hutt (60)], and with certain drugs, as arsenic [Jollos (61) and Jennings (62)], the antigenic poison, ricin [Gowen (63)], and endotoxins from *Salmonella typhimurium* [Hill, Hatswell & Topley (64)]. The breakdown of sugar metabolism in human beings shows similar familial differences; progeny of diabetics are 6.7 per cent diabetic, of nondiabetics, 1.2 per cent. This is attributed to as simple a cause as a single genic pair difference with diabetes recessive [Pincus & White (65)]. This gene expression occurs through its action on islets of the pancreas with the reduction of circulating insulin. The prolongation of life in the homozygous frizzle fowl by ample food supply and high temperature as against disability and death at low temperatures is a similar well studied case [Benedict, Landauer & Fox (66)]. Body heat loss and metabolic difficulties in meeting the caloric requirement are responsible causes of death. A similar significant adjustment to the environment, although the genetic factors can only be postulated, is that described by Dallinger (67). A flagellate having a temperature requirement of 60°F. was, in the seven-year period of experimentation, raised to a requirement level of 158°F. This change in temperature adaptation occurred in a series of discrete steps unequal both as to numbers of generations required and temperature rise withstood. Effects were also visible in a thickening of the cytoplasm. The results are interpretable as heredity reorganizations originating as progressive gene mutations in successive generations.

The effects observed above portray inheritance as leading to normal beings under one set of conditions and quite abnormal ones under the influence of another environment. Such environmental interactions have not often been reported, quite probably because of ignorance of gene physiology. When known, the reactions are specific for both gene and environmental agent. Specific gene substitutions in the inheritance will often give immunity to the agent.

Genic and Bacterial Interactions in Disease

In disease studies, as formerly conducted, it was customary to infect the experimental animal with massive doses of the pathogen. This may have been justified, for the objective was an animal sick to death. It did result in the view that the host constitution

played no part in the disease syndrome in those species where the disease was endemic. The conclusion was foregone, for only those animals which were completely immune could resist such treatment. Animals completely immune to massive doses, in general, do not occur in species which show susceptibility to a disease [Gowen (47)]. The result of such studies separated refractory from susceptible species but did not differentiate within species. Species differences depend on inheritance, but not the kind to which genetic methods can often be applied.

Recognition of the importance of dosage showed gradations from resistant to susceptible within species for diseases endemic to them. The scale of the effect was set by the dose of the pathogen, its pathogenicity, and the resistance of the host [Irwin (68), Schott (69), Lambert & Knox (70)]. The development of methods of analysis of disease variation permitted separation of these causative elements; this marked a significant advance in the understanding of epidemic and endemic disease. With severity of disease bearing a close relation to pathogen dosage, it became understandable why infectious disease mortality decreased in this and other countries with progressive improvement in hygiene.

The search for strain differences in animals is proving to be as profitable to the understanding and control of disease as it has been in plants. Reviews of the progress have been presented by Crew (71), Kozelka (72), Gowen (47), Hill (73), Perla & Marmorston (48), and Grüneberg (50), to mention but a few. Beginning with the Hippocratic Corpus, the prognosis of a disease was based on the constitution of the patient. By the fifteenth century it was concluded that many diseases have a hereditary element. From this time until the middle of the nineteenth century a fair number of structural diseases were traced to hereditary origins and lip service was paid to heredity in some infectious diseases, such as tuberculosis. The work of Pasteur and Koch demonstrating the importance of the pathogen led to a concerted attack on the significance of the hereditary factor. Bacteriology and serology provided new understanding of both the pathogen and the serum antibodies developed by the host. By a "house-that-Jack-built"-like process, this information was built into the acquired immunity concept, with both active and passive elements. This concept was used to minimize the possible effects of inheritance. But the fact that certain diseases, notably tuberculosis, leprosy, and various forms of cancer

annoyingly did not respond to bacteriological and serological knowledge, and the rejuvenescence of the study of heredity by vigorous research led to a re-examination of views, first for the host-lethal gene complex, then for the host-gene-pathogen complex. Tyzzer (74) recognized this relation in a study of the piliformis disease of mice. Susceptibility to this disease shows a species difference which may be analyzed because the species will cross. The study is incomplete since the numbers involved are not specified. In terms of case susceptibility, brown agouti mice showed two cases, the Japanese waltzer mice, one hundred and forty-four cases. The F_1, for possibly six mice, showed five cases: F_2, eleven; F_3, one; F_4, one; B.C. (F_1 to Jw), five; (F_1 to Br. Ag.), one; and ($F_1 \times F_2$), three. Gowen & Schott (75) extended the study; they showed similar differences with *Mus. musculus:* thirty-seven tested, no deaths; and *Mus. bacterianus:* waltzing (w), ninety-nine tested, eighty dead; Chinese nonwaltzer: one hundred and sixteen tested, thirty-two dead. The F_1 genes expressed dominance differently than in Tyzzer's work. The inbred lines, black-eyed white (bew) and silver brown (sb), behaved differently: w $\times$ bew produced fourteen dead of sixty-one; the w $\times$ bs, zero dead of twenty-eight. While Tyzzer seemed to demonstrate a dominance of susceptibility our work showed a partial dominance of the resistance for one cross and complete dominance for another. This is not particularly surprising, for the inbred lines differ, so the gene background is not the same in the F_1's. The w $\times$ Chinese cross showed two dead of sixteen. This indicates that the two bacterianus strains are not identical. The Chinese $\times$ Musculus gave zero dead of eighteen mice. The backcrosses showed segregation according to the strains entering the cross. The tentative hypothesis offered was that a single major factor difference may be largely responsible for the musculus, bacterianus difference. The w $\times$ Chinese crosses show that other gene differences must also exist. Susceptibility segregated independently of waltzing or dominant white gene pairs and sex.

Earlier work on human tuberculosis resistance by family studies, Hirsch 1883, Pearson 1907, and Goring 1909 [see Puffer (76)], culminated in indicating an exceptionally high degree of hereditary determination of both morbidity and mortality for this disease. This was checked by studies on inbred strains of guinea pigs [Wright & Lewis (77)]. The guinea pig is susceptible to the hu-

man tubercle bacillus. Inbred strains differed in survival, the average length of life varying from twenty-six days for line 32 to thirty-seven days for line 35. In crosses, high resistance was transmitted by both sexes. F_1's from high were better than high parent, indicating not only dominance but complementary effects. Susceptible inbred strains crossed were no better than the more resistant parent, or complementary effect was lacking. Thirty per cent of the variation in resistance in crosses was due to the blood of strain 35. Resistance factors were not correlated with sex or with such indicators of vigor as growth rate, adult weight, frequency or size of litter or per cent of animals raised to weaning. Lurie (78) observed differences in the survival value of families of rabbits; family F survived five months as compared to nine months for family A, but the data were on small numbers.

The human observations as extended by the epidemiological method of Ferguson (79), by the family study method of Pearl (80), and by the twin method of Diehl & Verschuer (81), Atzenhoeffer & Rife (82), Kallmann & Reisner (83), and others, all are concordant in emphasizing the significance of several gene pairs to susceptibility or resistance to tuberculosis.

Leprosy has a similar history. Hereditary susceptibility was recognized before the discovery of the bacillus, at which time the contagion view became dominant. This period was followed by one of difficulty in accounting for many phases of the disease on bacteriological or serological grounds. A better understanding of the mechanisms of heredity led to family studies and to the acceptance of hereditary susceptibility in a favorable environment as of primary importance in the occurrence of this disease [Hopkins (84), Aycock & Hawkins (85), and others].

Observations on diphtheria and scarlet fever resulted in analogous conclusions [Coca (86), Hirszfeld, Hirszfeld & Brokman (87), and Murray (88)]. Skin tests suggested that a part of the inheritance effect was expressed as presence or absence of natural antitoxins, a single gene pair being responsible for susceptibility and resistance [Rosling (89)].

Deep focal infections as exemplified by appendicitis have likewise been demonstrated to be of high incidence in some families and low in other pedigree lines [Cook (90), Baker (91), and Flavia (92)]. Predisposition to this condition is attributed to hereditary structural pecularities resulting in poor blood supply, poor con-

nection between appendix and digestive tract, etc. A parallel in the hereditary occlusion of the vagina, leading to similar changes and death, is found in mice [Gowen & Heidenthal (93)]. Focal infections of the udder in cattle also point to an hereditary basis for susceptibility [White & Ibsen (94), Ward (95)].

A more sharply defined case, where some of the pathologic as well as genetic factors are known, is that described by Hadley & Cole (38). A single recessive gene is responsible for susceptibility to generalized bacterial septicemia in epitheliogenesis imperfecta of cattle.

Brucella infection and resistance in swine were shown to turn on hereditary differences [Cameron, Gregory & Hughes (96)]. Extensive studies on rabbits [Manresa (97)] have indicated that susceptibility to brucellosis is characteristic of families with resistance partially dominant. Antibody immunity of previously infected susceptible females is transitory, whereas genetic resistance is transmitted to successive generations.

In bees resistance of the larval stage to *Bacillus larvae* follows genetic lines. By selection, disease incidence was changed from 75 per cent to nearly 0 per cent [Park (98)]. The inheritance expressed itself through instinctive activity for hive cleanliness in the resistant bees as well as possible antibodies in their blood.

One of the earliest and still unsurpassed works on hereditary effects in disease is that of Rich (99). Guinea pigs were infected with both *Pasteurella suiseptica* and *Salmonella cholerae suis*. Spontaneously occurring and inoculated cases were observed, with similar results in both instances. A single gene pair was found to be responsible for the defective formation of complement in the blood. The presence of two recessive genes resulted in this effect while either one or two of the dominant alleles gave full complement. Inoculation experiments with *S. cholerae suis* in one hundred guinae pigs resulted in 77 per cent mortality for the recessive phenotype, and 20 per cent for the dominant. As with many of the cases cited, the mechanism of the effects will be discussed in a later section.

The most extensive researches on host inheritance in bacterial resistance have been conducted with the rat, mouse, and fowl. F_1 crosses between strains, backcrosses of F_1's to each parental strain, F_2's, and further generations have been raised to establish the mode of inheritance.

Irwin (68, 100), working with inbred selected lines of rats, established strains which were 94 and 42 per cent susceptible when inoculated with one hundred and fifty million cells of *Salmonella enteriditis*. The F_1's were 55 per cent susceptible; the backcross to susceptible, 48 per cent; and F_2, 37 per cent. The F_1's and subsequent test generations may be somewhat low in susceptibility, due to some unknown cause, because further generations showed increased susceptibility to 66, 74, and 61 per cent. Measurable differences in season, environmental changes, or physical differences such as weight, age, litter size, or gain in weight of the animals could not account for these results. Hybrids between survivors and backcrosses to susceptible showed greater resemblance in mortality to the resistant than to the susceptible parent. These and other matings indicated that multiple genetic factors operating cumulatively and possibly with partial dominance were responsible for the differences in susceptibility. Genetic analysis by sire matings showed variations attributable to genetic factors which the inbreeding had not made uniform. Genetical and serological tests for the possibility that the transfer of active immunity had caused the changes in resistances of the different crosses were all negative. The cause of the susceptibility and resistance differences in the different rat groups is explicable by the postulated genetic factors.

Work at Iowa State College by Lambert (101) and Gowen (102) has clarified certain features of the typhoid syndrome of mice inoculated with *Salmonella typhimurium*. Schott (69) established the dosage mortality relation for mice. He tested six of the then existent inbred strains of mice with *S. typhimurium* by intraperitoneal injection. High susceptibility was observed, with 80 to 100 per cent deaths in the fifty-four to one hundred and eight mice tested in each group. A group (Schwing) from a dealer showed 82 per cent deaths. By selecting resistant parents, Schott increased the survival from 18 per cent to 36, 54, 60, 64, 68, and 75 per cent in six generations. F_1's between untested Schwing and silver had 22 per cent survival, and between selected resistants and silver survival was 63 per cent. A group of definite heritable genes for resistance had been concentrated in the selected lines. F_1 reciprocal matings of selected $\times$ silver, compared with the silver strain, showed that passive transfer of acquired immunity was not the explanation for the increase in resistance. Gowen & Schott (103), by double mating female susceptibles to resistant and susceptible

males in the same heat period, were able to obtain F_1 (susceptible × resistant) and pure susceptible in the same litters. These types were separable on the basis of coat color inheritance. Tests for typhoid resistance showed 47 per cent of F_1's survived and 0 per cent of (pure) susceptibles. This difference between F_1 and pure susceptible is expected if genetic differences are responsible for typhoid resistance but is completely contrary to any acquired immunity theory if immune bodies are to come from the mother.

Hetzer (104) followed Schott's selected strains for eight more generations of selection, the dose being changed successively from fifty thousand to two hundred thousand, two million, and ten million organisms. Survivors increased to 92 per cent, at which point they stabilized. Reciprocal crosses of resistant and susceptible showed that active or passive transfer of immunity was not a factor in resistance. Susceptible strains showed 2 per cent survival, F_1, 83 per cent; resistants, 89 per cent; and backcrosses to susceptibles, 49 per cent. Complementary genes for resistance were found in susceptible strains. There is no linkage of resistance genes with sex, albino, silver or agouti loci. Subsequent work by Zelle (105), Oakberg (106), Weir [see (107)] and Gowen (107) have demonstrated that six inbred strains of mice may retain their characteristic reactivity to typhoid for eleven years in a typhoid-free environment without any further selection.

Gowen & Schott (108) and Gowen (63, 109) showed that the constitutional basis for disease resistance was highly specific. The genes for resistance to the virus of pseudorabies, and for the antigenic poison, ricin, were not the same as those for typhoid and were independent of each other.

Webster (110), infecting by stomach tube with five million *S. enteriditis*, obtained characteristic death rates when he tested nine strains of mice, percentage of deaths ranging from nineteen to ninety-seven. From a random bred strain having 63 per cent survival, two major strains were isolated by progeny testing and then breeding from further untested progeny. The susceptible showed 18 per cent and the resistant strain 82 per cent survival after six generations. The effective selection occurred in the first two generations for the susceptibles and first generation for resistants. Pedigrees indicated that each line in general really traced to but a single pair of the original five hundred pairs. Reciprocal crosses of susceptible and resistant gave like results, with 82 per

cent survival; backcrosses to susceptible resulted in 52, 45, and 7 per cent survival (the 7 per cent is abnormal but possibly a chance result); backcrosses to resistant produced 70, 77, and 87 per cent survival; F_2, 65 and 66 per cent. These differences were carried over into mixed populations in that with spontaneous herd infection 70 per cent of susceptibles and 12 per cent of resistants died. The weights or number born or weaned did not account for these differences. Webster confirmed Gowen & Schott's observation that constitutional resistance to a disease was discrete; four strains showed different but uncorrelated degrees of resistance to louping ill (a virus) and to typhoid. Deaths due to *P. avicida*, *K. pneumoniae*, and the pneumoccocus were correlated with those of the *S. enteritidis*, indicating a common gene basis for resistance as well as a possible common mechanism.

Webster (111) further differentiated the typhoid susceptible (BS) and resistant (BR) lines with louping ill and St. Louis encephalitis virus (VS and VR) into four possible combination types: BRVR, BRVS, BSVR, BSVS. The F_1 crosses of the three last types were presented, together with F_2 and backcrosses. All the progenies followed what might have been expected from the separate inheritance of the genes for the different diseases if these genes resulted in partially dominant phenotypes. Unfortunately, the expected values for the F_2 and backcrosses were wrongly calculated, so that while two independent gene pairs may be all that are necessary to differentiate susceptibility and resistance for the two diseases in these particular strains, the proof is incorrect. For the general case covering more strains more gene pairs are certainly necessary.

Schütze, Gorer & Finlayson (112) and Gorer & Schütze (113) worked with branches of Webster's susceptible and resistant strains, Little's C57 black, and Strong's A_2. They confirmed that Webster strains are susceptible and resistant respectively. They also confirmed the conclusions of Gowen & Schott (108) and of Webster (111) that resistance to typhoid is uncorrelated with that for a virus. They demonstrated a fairly strong correlation between *S. typhimurium* and *S. enteritidis* resistance but lesser correlation with the resistance to *P. muriseptica*. Sex differences were noted for *S. typhimurium* but not for the other diseases.

Hill, Hatswell & Topley (64) selected for resistance to an extractable endotoxin from *S. typhimurium*. In ten generations

survivors among the selected reached 55 to 77 per cent; among unselected these numbered 9 to 19 per cent. Selected and control mice then tested with one hundred thousand living organisms instead of the endotoxin showed unexpected and unaccountable results. Selected mice survived no better than controls. They could only conclude that increase in resistance to toxin of selected generations was not due to specific antibodies.

Work on the fowl is as extensive and critical as that on the mouse. Two bacterial diseases have received most attention. Lambert (114, 115), Lambert & Knox (70), and Gowen & Bell (116) have studied the host pathogen relationship for *Salmonella gallinarum*, and Roberts, Severens & Card (117, 118, 119) that for *Salmonella pullorum*. Starting with random bred birds, Lambert showed that increasing dosage of S. *gallinarum* increases death rate. Random bred breeds differ in their resistance. Inbreeding selected survivors in the Leghorns resulted in an increase in resistance from 10 per cent to 85 to 90 per cent in four generations. Family lines differed in ability to transmit resistance. Reciprocal crosses showed that resistance was due to particular genetic constitutions and not to any acquired immunity. Genes for resistance were independent of the dominant white locus. Gowen showed that resistance remained constant even when the parents were not tested. Resistant and susceptible birds have not changed appreciably in sixteen generations.

Robert & Card (120) commenced their studies on the pullorum disease of chickens in 1924 and have continued to date. These selected strains were established by inoculating and breeding from the survivors the following year. In one hundred and twenty-eight tests over a ten-year period this selected stock showed 72 per cent survival, the control 27 per cent. F_1's, resistant $\times$ susceptible made reciprocally, were as resistant as resistant parents. Backcrosses $F_1 \times$ resistant were more resistant than $F_1 \times$ susceptible. From F_2 progeny it was possible to recover resistant and susceptible strains by selection. Repeated matings of a susceptible male $\times$ susceptible females produced less resistant progeny than when the same male was mated to resistant females. Similar matings of resistant male to susceptible and resistant females gave no difference in progeny. Resistance was the dominant phenotype. Acquired immunity was shown to play no part in results by both genetic and serological means.

Genic and Protozoan Interactions in Disease

Wenrich (121) has summarized the general host-parasite relationship for protozoa as follows: (*a*) there are many cases where the same or nearly related species of protozoa have invaded host species of widely different taxonomic groups; (*b*) closely related protozoan species may be found in the same host species; (*c*) species of protozoa belonging to widely different groups may be found in one host species. Such evidence points to mutational origin of the protozoan-host relation. These conclusions have widespread application to our theme, for if differentiation of parasitism occurs on a mutation basis, then differences should be separable through genetic means.

Well-worked-out cases of hereditary differences in the protozoan-host relation are few, possibly because they have not been sought for sufficiently. Cattle tick fever, caused by a piroplasma (*Babesia*), is a severe disease in unexposed American cattle, but Zebus are resistant. F_1 crosses are likewise resistant. F_2 and subsequent generations segregate for resistance. Two or more pairs of genes are necessary to differentiate the disease resistance of the two pure types. The inheritance effects are twofold: dislike of Zebus by the ticks (122) and increased resistance of the Zebus to the piroplasma (123).

Resistance of the mosquito *Culex pipiens* to becoming a carrier for avian malaria has been analyzed by Huff (124) as due to a single pair of genes, with resistance recessive. For another genus, *Aedes aegypti*, and for *Plasmodium lophurae*, Trager (125) established strain differences in susceptibility by selection but claims on rather tenuous grounds that inheritance is multigenic.

Harrison (126) reviews the adaptations of trypanosomes and *Borrelia* to antibodies produced by the infected host. These changes have been interpreted as similar to gene mutations in the protozoan, which are selected by the host antibodies. With a high rate of reproduction even low mutation rates of the protozoan genes in the direction of pathogenicity become important to disease resistance.

Genic and Helminthic Interactions in Disease

The first and still the most completely analyzed host-parasite genetic relationship involving a helminth is that of the rat cysti-

cercus disease due to the cat tapeworm, *Taenia taeniaeformis.* Eggs of the *Taenia* are ingested by the rat, pass through the stomach, and are freed of their shells in the small intestine. The free swimming morulae make their way into the portal blood stream and are filtered out in the liver capillaries. Here they form the cysticerci. As will be seen in a later section, analysis of this situation has furnished what is perhaps the outstanding contribution to our understanding of the initiation of cancer. Because of the interlocking nature of the all-important researches of the cooperating investigators, Curtis, Dunning & Bullock (127), the references will be cited together.

Eight inbred lines of rats were developed through continuous brother-by-sister matings from good dealer stocks of widely diverse origin, generations of inbreeding being one to twenty-one. The breeding led to a maximum of hereditary differences between inbred strains and a minimum within strains. The progeny of these matings were fed *Taenia* eggs at one and two months of age. A consistent age effect was noted; 62 per cent of 21,929 rats were infected at one month, and 44 per cent of 17,751 at two months.

Highly significant line differences were found, the per cent infection in different strains being: Aug., 33; Cop., 45; strain 230, 45; Aug. × Cop., 47; Mar., 52; Zim., 69; Fis., 73; and strain 344, 74. The inheritance of resistance to this large helminth was as pronounced as for any of the bacterial diseases. Crosses between the Aug. and Fis. strains showed 33 per cent infection. The backcrosses to the Aug. showed 29 per cent and to the Fis., 38 per cent infection. The data reveal that genetic resistance to the parasite is dominant over susceptibility. It segregates in the backcrosses, crosses to resistant Aug. being even more resistant than the resistant strain, crosses to the susceptible being still quite resistant although approaching the susceptible stock. These hereditary effects were complex, depending upon several gene pairs. The hereditary effects were analyzed still further; for rats fed at one month the males were 13 per cent more susceptible than the females, and at two months, 21 per cent. This difference shows the cumulative effects of gene dosage for sex-linked genes.

Ackert (128, 129) has reviewed his work on breed resistance of the fowl to *Ascaridae galli.* Nutritional condition is important but under uniform conditions such genetic differences as those which separate the heavy breeds, Rhode Island Reds and White

and Barred Plymouth Rocks, from the White Leghorn and Minorca led to a significantly greater resistance in the heavy breeds. Similar but less exact studies on sheep have led to the isolation of similar breed differences in relation to two or three genera of nematodes.

Genic and Viral Interaction in Disease

Examples supplied by plant diseases are among the more critical and informative illustrations of the import of the genotype on the disease syndrome. This is particularly true for the virus diseases [Gowen (107)]. Linked with the plant data, the observations on virus diseases in animals become very suggestive if not clear-cut proof of the importance of genetic determination of resistance. Rous (130) was able to isolate a virus, which caused tumors in most of the fowl inoculated. Banting (131) has inoculated random bred Barred Rocks with tumor transplants containing both virus and tumor cells. Of these, about one in one hundred and sixty-four was resistant and one in fifty showed some degree of resistance. A study was also made using the Fujinami tumor. Of one hundred and fifty-eight birds, twenty-two showed resistance, 1:8 as compared with the Rous 1:51. Birds resistant to the Fujinami were somewhat more resistant to Rous tumors than untreated birds; twelve resistant birds were inoculated and but two survived. Rous resistant birds developed tumors and died when treated with Fujinami. Three rather important suggestions came out of this work even though the host material is randomly distributed for genotype: (*a*) resistant and susceptible individuals are found in this population as one might expect on inheritance grounds from its mixed genotypes; (*b*) the resistants are graded from clear resistants to clear susceptibles; (*c*) resistance to one tumor complex is not identical with that to another. These facts parallel those which were earlier reviewed, where a bacterial species was the pathogen. Banting (131) shows that the results for virus and tumor cells are not the same and that humoral antibodies do not account for the genetic results, but more of that later.

Transmission of a virus disease through an insect vector is not simply a means of bringing about contact. This is well recognized in insects which require an incubation period before transmission is possible. Storey (132) developed by selection two strains of

Cicadulina mbila, one able to transmit streak of maize (active = A), the other not (inactive = N). Progeny of crosses were as follows: A × A = A; N × N = N; N♂ × A♀ = F_1 (40 A♂ + 46 A♀); A♂ × N♀ = F_1 (77 N♂ +82 A♀); F_1 A♂ × F_1 A♀ = F_2 (12 A♂ + 10 N♂ + 24 A♀); F_1 N♂ × F_1 A♀ = F_2 (38 A♂ + 23 N♂ + 29 A♀ + 43N♀). These results led to the surprisingly simple interpretation that the ability to transmit this disease was due to a sex-linked dominant gene. Chromosome-sex relation of this species is not known to us.

Genetic differences in resistance to virus diseases were demonstrated by Gowen & Schott (108). Inbred strains of mice were inoculated intraperitoneally with 20 mg. of rabbit brain containing pseudorabies virus. The observed percentages of survivals were S 8, Ba 12, Sil 52, and Wf 55. F_1 crosses of S × Sil showed intermediate survival of 24 per cent. The inheritance differences between the two inbred lines led to an intermediate genotype in the hybrid. The S mice were highly resistant to the bacterium, *S. typhimurium;* the Ba, Sil, and Wf were highly susceptible. Webster (111) selected for louping ill and St. Louis encephalitis virus in mouse strains resistant and susceptible to *S. enteriditis*. He was able to establish both resistant (VR) and susceptible (VS) virus lines in each strain of mice, VR having 85 and VS 4 per cent survival, the F_1's 65, and backcrosses to VR 82, and to VS 52 per cent. These results confirm those of Gowen & Schott (108) in the lack of dominance of resistance in the F_1 phenotype. Comparison of typhoid resistances to virus resistances between these strains likewise confirms Gowen & Schott's data, in that independence is observed.

In horses, Lambert, Speelman & Osborn (133) observed breed differences in an outbreak of encephalomyelitis in yearlings. Nonius breed had 50 per cent incidence to 11 per cent in horses of other breedings; in foals Nonius had 73 per cent to 11 per cent for the others. There were no sex differences, but in contrast to most other diseases host resistance was recessive.

With human poliomyelitis Addair & Snyder (134) present a family study in which 29 cases of the paralytic type appear. Similar family differences for this disease are cited by Taylor (189), Stephens (190), and Aycock (191). The explanation suggested by Addair & Snyder is simpler than that found for the better worked out cases of the host-virus relation with other viruses and animal

species, i.e., an autosomal recessive of 70 per cent expression for susceptibility to paralytic poliomeylitis.

In insects Black (135) tried to establish two strains of *Aceratagallia sanguinolenta*, one able, the other unable, to transmit potato yellow dwarf. Ten generations of selective breeding failed to eliminate all unables from the able strain or vice versa. The strain differences seemed clearly inherited, but through several genes and with a fair degree of environmental influence.

In fowl, Hutt & Cole (136) during a period of ten years of selection were able to separate strains with 20 to 30 per cent differences in survival. The reduction in deaths is largely attributed to lessened lymphomatosis. These results are a confirmation of earlier observations by Asmundson & Biely (137), Biely, Palmer, Lerner & Asmundson (138), and others.

The Gene and Its Interaction with Agents of Diseases of Obscure Etiology

For want of a better classification cysticercus tumors, spontaneous tumors, leukemias, rheumatic fevers, and epidemic disease in oysters, and the composite of factors which lengthen or shorten life will be included in this section.

Early in the century Loeb (192) and later Tyzzer (193) and Murray (194) pointed out that breast cancer in mice and human beings tends to follow family lines. In 1916 Maude Slye (195) showed like results but made what is surely a false deduction, for while cancer certainly follows family lines, all cancer is not to be lumped together in a single category nor is all cancer due to a single recessive gene. Cancers fall into many categories differing in origin, in type and, when inheritance has been studied, in gene pairs and environmental factors, as shown by Curtis, Dunning & Bullock (139 to 145), Lynch (196) and the Jackson Memorial Laboratory group (197), among others.

The outstanding mammalian cancer investigations, commenced in 1917 by Bullock & Curtis with Dunning (139 to 145) joining the group in 1926, gave the first clear-cut evidence that a living agent, the cysticercus of the cat tapeworm, may be responsible for initiating cancer. By establishing eight inbred lines of rats they showed that the susceptibility to cancer deaths in these lines was inherited from one generation to the next and that this inheritance depended on more than a single gene pair. The inheritance effect

was similar to that noted by other investigators both before and since; it gained value, however, because it was based on large progenies (52,223 in 1932) with each animal having a complete necropsy. This work stands out in the respect that the inheritance effects were analyzed for the character basis responsible for the observed inbred strain differences in cancer incidence. Two inherited familial characteristics were shown to raise or lower the probability of the occurrence of cancer; the first was concerned with the longevity of the strain and the second stimulated resistance to the invasion of the intestinal tract by the *Taenia* larvae. Once the cysticercus developed in the liver the generation of a tumor was a matter of time, whatever the strain of rat. Cysticercus cysts were distributed in the lobes of the liver approximately in proportion to the weight of the lobes, so were the tumors. It was a matter of chance which normal cells touched by the irritant became cancerous. These results were checked by the use of the carcinogenic hydrocarbons. For both cysticercus and hydrocarbons the dosage was a prime factor in the time interval required for a cell, out of the many cells exposed to risk, to become tumerous. The very low frequency of cancer cells, considering the number of cells exposed and the random point mutation-like distribution of the cancer cell among the rest, may be interpreted as a gene change in a locus important for growth control. It is certainly true that when a cell once loses its growth control it transmits this condition to its descendents as long as it remains in a suitable environment. Finally, it has been possible to duplicate the tumor propensity of the living cysticercus by extracts of the ground and centrifuged larvae.

Genetic specificity in the etiology of spontaneous rat tumors not related to the cysticercus agent and independent hereditary transmission of factors playing a determining role were demonstrated by Curtis, Dunning & Bullock (146). Forty-seven lymphosarcomas out of fifty-five appeared in one inbred line; three hundred and sixty-three thymic tumors out of four hundred and fifty characterized the other line. F_1 has no thymic neoplasms, and mesenteric lymph node tumors were less than in the first parent strain. Both categories of tumors appeared in the backcrosses to each of the parent strains.

Furth (147) has presented a recent review of leukemia research. MacDowell and his associates in their very significant

work, as recently summarized (148), bring out some important parallels between these neoplasms and diseases of bacterial origin. The leukemic cells of the mouse, like bacteria, when introduced into a suitable mouse strain have the capacity to invade all tissues. Separation of the individual cells in reproduction gives the huge numbers of discrete cells which are necessary to uncover the rare changes in the cell characteristics and to separate them from unchanged types. This is another parallel with bacteria and protozoa. MacDowell demonstrates the consistent pathogenicity of four transplanted leukemic lines over fifty generations of passage. The result is like that observed by Zelle (105) in passage of *S. typhimurium* through strains of susceptible and resistant mice for thirty-two generations. In both cases the host and pathogen were in stable balance. When a spontaneous tumor is first passed it normally has an incubation period between inoculation and death one to five times that attained after ten or fifteen passages. During these passages the leukemia kills progressively more and more rapidly until the asymptotic point is approached, when virulence changes but slowly. The mixed nature of the cells in spontaneous tumors may possibly account for the virulence increase as a selection of the most adapted type out of all the rest for that particular host. This could occur without change of fundamental genotype, for cytoplasmic effects impressed on the cell by genotypes other than its own may persist for a fair number of cell generations; this is seen, for example, in the direction of the spiral in the snail shell and sex ratio genes. After a rather steady death rate for many passages a leukemia may suddenly increase in virulence, as witnessed by lesions displaying greater toxic effects and a general shift toward a larger celled leukemia. These changes may not be synchronized, so that each may be a separate event.

Changes of this type have also been observed by Zelle, Lincoln, Lincoln & Gowen, and Bell & Gowen (105, 150, 107, 149) in typhoids of mouse and chicken and in *Phytomonas* wilt in corn. Much less frequently a sudden loss of virulence occurs in the leukemia line. This occurs, too, in *Phytomonas* passed through susceptible inbred corn hosts. It has not been observed in the typhoid organism when in either resistant or susceptible mice but has occurred several times on culture media. In the bacteria the shift in virulence appears due to the selective pressure of the medium, the resistance or susceptibility of the host, or test tube nutrients.

In leukemia, since the C58 mice are constant, a parallel explanation would need some added modification, such as that the suddenly appearing lower virulence cells blocked out the symbiotic relation of host cells to the more rapidly fatal leukemic cells. That host differences may occasionally play a part in accompanying leukemic cell changes similar to those observed in the bacteria is shown by tumor 9, derived from line 1 by intervention of foreign strain normal cells injected earlier into the susceptible host. The long-time observations on leukemic lines, more than one thousand four hundred passages in one case, have brought out the slow but directional shift toward virulence after a line has reached a killing level of six to ten days. As MacDowell (148) points out, the small interval of these successive shifts might have been large if the killing time of the line had been greater. The damping effect of approach to the physiological limit for time of death has its effect. Two points stand out: changes are fortuitous, and large initial changes are followed ordinarily in continuous series by sudden changes.

The C58 host appears to exert a continuous selective pressure for the rapidly killing leukemic cells. Similar effects are noted in the mouse host to *S. typhimurium*. Mixed cultures of virulent and avirulent lines of typhoid in one mouse passage will come out without a single avirulent organism. We have never been able to perform an experiment with mouse typhoid where there was even an approach to monohybrid ratios for resistance and susceptibility. MacDowell (148) has done just that for leukemia, and the lessons to be learned from that and following experiments are significant. In the interval from twenty-three to one hundred passages C58 was 100 per cent susceptible to line 1 leukemia, StoLi mice were 0 per cent susceptible, F_1 all susceptible, and BC to StoLi 50 per cent susceptible. Evidence for monohybrid inheritance is good. In the one hundred and forty-third passage, when the line was killing C58 in five days instead of the previous seven to nine, the genetic expectations were no longer met, as line 1 killed more than expected. By the one hundred and sixtieth passage 44 per cent of the resistant StoLi were killed and by the two hundredth passage deaths approached 100 per cent.

This latter type of result was paralleled by the writer's inbred strains tested for mouse typhoid. Our standard line of bacteria has a relatively fixed kill for a given host; our most virulent line has

relatively a fixed kill for these same hosts but is in every case higher. Similarly, our avirulent line, also derived from the standard, has a fixed kill for the lines but smaller than the standard. Effects of dosage changes lead to comparable changes in both the leukemic and bacterial disease syndromes.

Another disease in which the etiology is not entirely clear and the genetic constitution strongly influences the morbidity is rheumatic fever (151, 152, 153). In two generations of families of affected children Gauld, Ciocco & Read (153) found 3.7 times the incidence of rheumatic fever that was found in the control group.

In sea animals also, inherited resistance plays a part in overcoming an introduced endemic. Up until 1915 the Prince Edward Island oyster beds were relatively disease free. From 1915 to 1930 the beds were progressively infected and wiped out. Resistant oysters appeared in one small locality. These replaced the susceptible strain and on transplantation were shown to be equally resistant in other localities. As Needler (154) points out, control of this infectious disease was possible even though the etiologic agent was not fully established.

A composite of these resistant factors makes what we, and the Hippocratic Corpus twenty-five hundred years before us, call a good constitution. Such gene collections are frequently grouped together and purified in inbred strains. They sometimes appear by chance as family characteristics in such random mated groups as the human being. Dunning & Curtis (139, 145), as a sideline to their outstanding studies on cancer, have made a study of these differences in inbred rats. The inbred rat strains had average lengths of life in months, as follows 11.1, 11.9, 12.3, 13.5, 13.6, 14.0, 18.8, two F_1 hybrids, 16.8 and 21.7; and two backcrosses, 15.1 and 16.4. The genetic constitution increased the life span 70 per cent. In another experiment they crossed the Fis. strain, with an average life of 11.4 months, with the Cop. strain which averages a 17.9-month span. The F_1 was like or superior to the longest lived parent, 17.7 and 20.9 months. Three genetically different backcrosses to the long lived parent gave comparable results, 19.9 months; two to the short lived parent resulted in progeny with average survival of 14.5 months. The significant effects of heredity are evident. F_1's show dominance of the survival with some heterosis in the cross to the short lived female strain. This precludes a maternal effect. The backcrosses show segregation and heterosis

over what might be their expected survival value. The major pathological changes in the lines were acute pneumonia, 70 per cent; ulceration of cecum, 14; peritonitis, 10; acute enteritis, 9; spontaneous tumors, 3; chronic arteritis, 2; jaundice, 1; and interior abscesses, 0.6. The multigenic inheritance for survival opposes these diseases. To a limited degree Pearl (198) has shown similar results in human families.

Inherited Modifications in Pathogens and Their Effects on the Host Disease

There can be little doubt that Koch's insistence that bacterial species do not vary did a real service for bacteriology when the emphasis was properly on identification of the pathogen. But bacteria, like other living things, do change. By 1904 Barber developed his micropipette, by which single bacteria could be isolated and the characteristics of the single progenies could be observed with certainty. The conclusions he drew, adverse as they were to Koch's contention, have not been altered by later work (155). Out of many thousands of observed bacteria *B. coli*, he selected one hundred and ninety cells on the basis of morphological differences from the parent coli type. Most of these cells failed to develop clones. Many were environmental variants which returned to type, some were unstable, some were quantitative rather than sharply different qualitative variants, and three met all the criteria for mutant types. His conclusions have been abundantly confirmed by later workers. Variations give rise to races permanently different in morphology. They arise suddenly and independently and are to be compared with mutants observed in higher plants.

These observations have been extended to both the protozoa and viruses. Some change in the inheritance mechanism as stable as that of a mutant gene may take place in the organism. Stewart (199) has studied the lactose fermenting properties of coli-mutabile; Lincoln & Gowen (150), the mutation of color and colony characteristics both under natural conditions and with x-ray exposure. Estimates of the rates of change both spontaneously and with x-rays have been presented for bacteria and viruses by Gowen (156) with results which were comparable to those obtained in proven gene systems.

Two types of changes appear. One is stable as measured by

long passage through many generations of progeny. The other is unstable. These two types are widely separated in the frequency of their mutant progeny. The first type has the frequency of gene mutation approximately 1 in 10^8. The second has parent type progeny to aberrant type in ratios of 50:1 to 1000:1. The aberrant type is not random but is specific for the particular parent. When it occurs the aberrant type is stable while the parent type continues to have progeny in about the previous ratio [experiments of Zelle (105) and Lincoln & Gowen (150)]. To the writer this latter type has strongly suggested somatic crossing over or the chromosomal rearrangements which bring about the unknown phenomenon called mottling as the source of the variation, rather than gene mutation.

The possibilities of these hereditary modifications when introduced into the disease syndrome lead to strikingly significant modifications. Wellhausen (157), working with inbred lines of corn, showed that if mixed virulent and avirulent organisms were administered to genetically susceptible hosts these selected in favor of the avirulent mutant type of organism. Genetically resistant hosts selected for the intensely virulent mutant forms. Lincoln (149) confirmed these results, extended them to other inbred strains and performed critical experiments with mixed avirulent and virulent cultures where the two types could be distinguished on genetic grounds. In these mixed cultures the avirulent were selected for by the susceptible host, the virulent by the resistant.

Zelle (105) performed like experiments but with somewhat different results. Both the susceptible and resistant mice selected heavily for the virulent organism. Culture media, on the other hand, seemed to favor the avirulent type. Other experiments in our laboratory show that with *Salmonella gallinarum* the culture medium is rather sharply selective for the avirulent type, while again both the susceptible and resistant host strains are antagonistic to the avirulent forms.

These results have striking consequences to epidemic disease and to the treatment of infections. Leete (158), in a bacteriologic study of diphtheria in Hull, England, was struck by the constancy of occurrence of intermedius type over a period of many years. Superimposed on this type he saw the toxic E.C.4 strain appear, become dominant, and disappear. The gravis type rose to cause a fatal-

ity rate of 15.9 per cent in 1938, followed by a gradual decline to 2.6 per cent in 1943. A slow decline was noted for the intermedius. Susceptibles were selected out of the host population and a flare-up of the disease came with an organism capable of overstepping the genetic and acquired resistance mechanisms. The same facts were more critically brought out by Zelle (105) when *S. typhimurium* avirulent and super virulent strains, derived by mutation, were introduced into susceptible and resistant hosts. The six host strains tested kept their relative resistances; the avirulent killed only a few in the susceptible strains, and the virulent killed all but a few in the resistant strains. These results and others make it appear that in order for an epidemic to occur the pathogen must be different from the endemic type present in the population. The reproductive possibilities of the aberrant form, whether mutant or introduced, are so great that such situations need not be very rare. The host genetic variation can and will modify the result, but only slowly as compared to the possibilities of a really virulent organism in a mixed population. Environmentally the hygienic possibilities of changing the epidemic course of a disease are great in so far as the dose reaching the host is reduced.

Similar forces are at work in the course of a disease within an individual. The antigenic poison, ricin, is capable of exerting selective activity upon genotypes resistant to it [Gowen (63)] and this is but one of several drugs with which this is demonstrably true. Plough (159), working with *Staphylococcus aureus*, demonstrated that an inherited resistance was attained in more than 40 per cent of organisms isolated from lesions of patients treated with penicillin. *In vitro*, most but not all lines which have been exposed to penicillin become resistant. Sensitive lines may become resistant after forty-eight hours of exposure. Human and mouse studies suggest that this may occur *in vivo*. There is some evidence that resistant lines may become susceptible after a lapse without exposure to penicillin. The replacing of susceptible by resistant staphylococcus was demonstrated by Demerec (160) when the organism was exposed to penicillin *in vitro* and (as in similar earlier work on bacteria) the response was mutative in nature. Oakberg & Luria (161) have demonstrated a like effect of the sulfa drugs for the same pathogen. The inherited resistance to one drug is independent of that to the other drug, a demonstration of the specificity of the inherited resistances comparable to that in gene behavior in

organisms where this effect may be analyzed genetically. The data on change from susceptible to resistant for other drugs and environmental stimuli (bacteriophages, heat, etc.) goes back several decades and is equally convincing.

The importance of these transmitted changes to protozoan disease has equal significance to that cited above, but as they have been reviewed in Volume I of these Reviews by Harrison (126) they will be omitted here.

The Gene and the Physical Basis for Its Effects on Natural Resistance in the Host

The series of phenotypic changes induced by genes causing pathological conditions as described in section 1 have not been worked out in most cases. For the minority group Grüneberg (50) has presented an extensive and informative summary. The case of the dwarf mouse of Snell (7) will clarify the trend. The dwarf condition is due to a single major recessive gene. The gene expresses itself in multiple effects; homozygous recessive animals are one-third to one-half normal size, are sterile, show thyroid, adrenal and reproductive repression, and the basal metabolism is reduced to one-half normal. The pleotropic effects were traced by Smith & MacDowell (162) to the pituitaries, where the eosinophil cells were shown to be nearly lacking. Transplantation of pituitaries from normal mice caused a resumption of growth and a return of thyroid, adrenals, and reproductive tract (save for the female) to normal. Further study by Bates, Laanes, MacDowell & Riddle (188) showed that anterior pituitary extracts from cattle—prolactin and an ethanol precipitate—when recombined would duplicate the effects of the normal pituitary. The phenotypic pleotropic effects of the gene were shown to be due to effects on a single type of cell which, in turn, elaborated pituitary hormones. Replacement therapy with products of the normal dominant allele led to the return of the dwarf mice to normal in size and other features. The same type of replacement therapy leads to a repair of defective inheritance in the individual: anemia in mice (163), diabetes in man. The return to normal does not alter the defective inheritance transmitted to the next generation.

A similar chain of events occurs in infectious disease in guinea pigs. Rich (99) showed that pigs dying in an epidemic were largely susceptible because they lacked complement in their blood. This

complement lack was due to a single recessive gene. The mechanism by which such genes cause their effects has been elucidated in part by the work of Beadle, Tatum and their associates as a one to one relation between the required gene and the precursor to form the product next higher up in the chain of necessary substances leading to the ultimate phenotype [see Beadle (164) for extensive reviews].

In the above case the resistance of guinea pigs to *S. cholerae suis* was likewise due to the formation of complement by the same dominant gene. A similar but less well worked out case genetically is resistance of the fowl to helminthic infections. Duodenal mucus contains an antihelminthic substance. This is not an antibody in the serological sense, since it stands autoclaving [Ackert (129)]. Resistance in bees to fowl brood established by selection has been shown to be due to inheritance of instinct for hive cleanliness [Park (98)].

Since the discovery of the passive transfer of immune bodies either through the placenta or by the colostrum to the progeny, it has been a favorite hypothesis of some that all inherited resistance to disease was along this path and not in any sense genetic. The acquired immunity hypothesis was an intriguing one, generally demonstrated by the passage of some antibody developed against a foreign protein. How close this demonstration might be to the conditions of infectious disease was generally not investigated by the proponents. Geneticists made this investigation in a large series of critical tests. Lambert (165) demonstrated with *S. gallinarum* that reciprocal crosses of resistant and susceptible fowl gave the same degree of resistance in F_1 progeny. Resistant mothers that were carriers of *S. gallinarum* had progeny which were equally as resistant as those from noncarrier mothers. Similar results were attained by Roberts & Card (117) in their studies on host resistance and susceptibility to fowl pullorum. For the rat-typhoid relation, Irwin (100), with reciprocal crosses of resistant and susceptible strains, showed that susceptible mothers had a lower rate of mortality than resistant mothers. Noncarrier females, recently recovered from the disease, had progeny no more resistant than those from the same females some months later. Females with low or no agglutinins had progeny practically as resistant as females with one-twentieth or above titer. Sera from resistant and susceptible unexposed rats mixed with *S. enteriditis* showed no

differences in protective value. On the other hand, sera from rats surviving typhoid, taken three weeks after inoculation, showed some protection. Carriers had progeny slightly more susceptible than noncarriers and showed more families with 100 per cent deaths. Progeny of carriers and noncarriers showed resistance only if they came from resistant families. All of these results are contrary to the expectations of the hypothesis of acquired immunity in accounting for the strain differences in virulence. Schott (69) tested for any transfer of acquired immunity to *S. typhimurium* to the progeny by reciprocal matings and made comparisons of selected line differences in mice without finding any effects. Differences were genetic. Gowen & Schott (103) obtained F_1 resistant $\times$ susceptible progeny and susceptible progeny in the same litter from susceptible mothers. The F_1 progeny were partially resistant; the pure susceptible were all susceptible. These results are fully expected on genetic transfer of resistance, but are totally contrary to the hypothesis of acquired immunity. Hetzer (104) compared reciprocal crosses of two susceptible strains with a resistant but could find no evidence of passive transfer of immunity. Again the results met the genetic expectations. Webster (110), by selection of progeny from unexposed parents, was able to differentiate a mouse population into susceptible and resistant strains. As *S. enteriditis* was not present, passive or active transfer of resistance to this organism was an impossible explanation of the results. Gowen & Schott (108), Gowen (102), Webster (111), and Gorer & Schütze (113) have shown that mice within inbred strains display characteristic resistance or susceptibility to a disease without having had previous exposure to it. These strain differences were found for diseases caused by the following pathogens: *S. typhimurium*, *S. enteriditis*, pseudo rabies virus, *Pasteurella avicida*, *B. friedlaenderi*, *Diplococcus pneumoniae*, and louping ill. Genetic differences to chemical poisons have been observed: in mice, for ricin (63) and endotoxin extracted from *S. typhimurium* (64); in scale insects, to hydrocyanic acid, methyl bromide and ethylene oxide [Quayle (166)]; and in flies, to DDT.

The investigations are all against passive and active transfer of immunity as an explanation for observed inherited resistance to disease. The evidence has now become so extensive that those who still insist on this hypothesis may rightly be asked to furnish experimental evidence in its support.

The genic relations in the hereditary mechanism for resistance were investigated by Gowen & Schott (63, 108) for diseases due to *S. typhimurium*, pseudo rabies virus, and the antigenic poison, ricin. The gene loci required for resistance and susceptibility to these three diseases were independent. Webster (110) collected data on inherited typhoid resistance or susceptibility with four other diseases for four strains of mice. His conclusions are not quite borne out by the data, so the correlations as calculated by the writer are presented. Interpretation is difficult for data on so few strains. Strain differences in resistance to *P. avicida*, *B. friedlaenderi* and pneumococcus are highly correlated. Typhoid resistance is closely correlated with *Pasteurella* resistance (0.7), moderately with *Klebsiella* and pneumococcus resistance (0.5), and hardly at all with louping ill (0.04). Louping ill resistance is moderately correlated with *Pasteurella* (0.7), moderately with *Klebsiella* (0.5), slightly with pneumococcus (0.2), and not at all with typhoid resistance. Interpreted in terms of gene effects these correlations could mean that in the inheritance some genes could have common effects on resistance to *Pasteurella*, *Klebsiella*, and pneumonia, but that the genes for typhoid and louping ill resistance were independent. As louping ill and St. Louis encephalitis viruses were used interchangeably with apparently similar results, resistance to each would appear to be controlled by the same genes. The independence of typhoid and the viruses, louping ill and St. Louis encephalitis, in resistance was borne out by Webster's establishing the possible four lines by selective breeding, BSVS, BSVR, BRVS, BRVR

Schütze, Gorer & Finlayson (112) performed a like experiment using Webster's susceptible and resistant strains and two others. Dosage was such that a good differentiation for *S. typhimurium* and *S. enteriditis* was not obtained. Differentiation between the four strains was not great for louping ill, *Pasteurella*, or pneumococcus. Results confirm a correlation between *S. typhimurium* and *S. enteriditis* resistance. Strain resistance may be moderately correlated with *Pasteurella* resistance but not with pneumococcus or louping ill.

Hill, Hatswell & Topley (64) compared resistance to an extracted endotoxin of *S. typhimurium* with resistance to the intact organism but found no correlation between the two. This strange result taken at its face value would indicate that resistance against

S. typhimurium in mice is something apart from the endotoxin generated by it, the genetic basis for each being independent.

For transplantable leukemia, which, like transplantable tumors, is in many respects more like the host-pathogen problem than that of the initiation of spontaneous tumors, MacDowell (148) has shown that the gene arrangement of host and pathogen may change with the passage of many cell generations. Similar differences have been noted for the typhoid diseases [Gowen (102, 116)], differences which are attributed to mutation in the pathogen and selection of the mutant type.

Humoral mechanisms have been suggested as the character bases on which inherited immunity may rest, because of the demonstration that agglutinins, precipitins, and bactericidins develop in the course of active contact with a disease or with immunization. Some of the earlier work, particularly on diphtheria, gives support to this contention. Hirszfeld *et al.* (87), Murray (88), and Rosling (89) each attribute resistance to the natural presence of or ability to develop antitoxin early in the initial phases of the disease. Basu & Roy (167) support this view as accounting for the natural presence of antitoxin in some horses. Landauer (168) draws attention to a similar parallel of increased antibody titer in gravid and lactating women in the absence of antecedent contact with dysentery, anthrax, and poliomyelitis.

Shrigley & Irwin (169) have demonstrated that the sera of the normal ox, rabbit, and sheep are highly bactericidal for *Brucella suis*, while guinea pig serum is not. Guinea pig complement would not activate the normal thermostable bactericidal fraction of ox, rabbit, or sheep blood. Rabbit complement activated only the rabbit factor; ox complement acted with rabbit and ox bactericidal fractions, but only partially with that of the sheep; sheep complement acted with sheep and rabbit factors and was nearly as good as ox complement for ox factor. These differences are attributable to inherent differences between species. Similar species differences in bactericidins have been found for the typhoid organisms in our own work. Irwin & Hughes (170) have shown that the whole blood of rats resistant to *S. enteriditis* has greater bactericidal power than that of rats which succumb. The experiment was so arranged that either the serum or the cells could be the responsible agent. We are informed that the serum interpretation is pre-

ferred. In view of our extended and fruitless search for differences in bactericidal properties of sera from resistant and susceptible mice and fowl, we are inclined toward a cellular explanation. Bull & Tao (171) concluded from their studies that the bactericidal power of human whole blood is due to phagocytes. Cameron, Gregory & Hughes (96) selected against hogs containing agglutinins for brucellosis in selecting for resistance to this disease. Roberts, Severens & Card (118) found no difference in the bactericidal power of chicks resistant and susceptible to pullorum.

Work in our laboratory (105) has demonstrated that mutant types of organisms are distinguishable from the normal type in many ways. A single mutant may have simultaneously changed in a morphological character as well as in its virulence. By inoculating a mixture of two distinguishable types it has been shown that the host exerts a tremendous selection on the two types, permitting only the most virulent to survive. Metchnikoff (172, 173) pointed to the reticulo-endothelial system as the active element in resistance, while Murphy & Sturn (174) point to the lymphocytes as a significant part in the system of immunity to transplantable tumors and to bovine tuberculosis. Maximow (175) gives emphasis to this view by his microscopic observations on transformation of blood lymphocytes into macrophages, a view which has received further support from Taliaferro & Klüver (176).

Roberts, Severens & Card (118) related pullorum resistance in their strains of chicks to both leucocyte and lymphocyte behavior by showing, in the period of susceptibility from hatching to ten days of age, that the resistant strains had larger numbers of leucocytes at each age, that leucocytes increased rapidly with age, and that the lymphocytes increased from 5 to 65 per cent of the total leucocytes. In *in vitro* tests lymphocytes showed phagocytic activity at 37.5°C. (119). Spleens of resistant chicks were larger than those of susceptible. Splenectomized resistant chicks had a notable reduction in lymphocytes and a corresponding reduction in resistance. Lymphocytes were more numerous in the arterial sheaths of the spleens of resistant than of susceptible chicks. These results suggest the spleen as the organ of defense in pullorum.

Reich & Dunning (177) in studies on six long inbred strains of rats showed that the ability to withstand various diseases and environmental conditions with resultant longevity is highly related

to increasing numbers of leucocytes. The neutrophils form a higher proportion of the leucocytes in those rat strains with best survival fitness.

Gowen & Calhoun (178) in studies of blood cells of two hundred and fifty mice composed of about equal numbers of six strains demonstrated the inheritance of both characteristic erythrocyte and leucocyte levels. The numbers of leucocytes were directly related to the typhoid resistance of the strain, the higher the numbers the greater the resistance. The proportion of the different leucocytes was not important. These facts are a further confirmation of the unitarian view for the origin of these cells.

Amoss, Taylor & Witherbee (179), by treating monkeys with x-rays, reduced lymphocytes and resistance to poliomyelitis concordantly. Roberts, Severens & Card (118) also lowered the number of lymphocytes and resistance of their chick strains to pullorum when they x-rayed them. Gowen & Zelle (180) x-rayed 789 mice belonging to six strains ranging in resistance to typhoid from 8 to 86 per cent. When contrasted with their 468 unirradiated controls, increasing x-ray dosage was found to decrease resistance at a simple exponential rate. This rate was the same for each strain. However, as is well recognized by workers in the field of radiant energy, x-ray or heat may materially affect the animals in many other ways in addition to leucocyte number [Perla & Marmorston (48)]. Our experiments on mice, where the quantitative relations on the six strains are worked out, speak more strongly for the capacity of the animal to form leucocytes as one of the inherited characteristics significant to natural resistance to typhoid than those experiments which are based on but two lines.

Oakberg (106) showed that the characteristics of the macrophage system form an important part of the basis for natural resistance. He confirmed the observations of Seiffert *et al.* (200) and Ørskov *et al.* (201) that the liver and spleen filter out most of the invading typhoid organisms in mice. Strains of mice differing in their typhoid resistance react very differently to the presence of these bacteria. Intermediate and highly resistant strains show severe liver and rare splenic lesions. Susceptible strains have small or no hepatic necrosis. Splenic macrophages in intermediate and highly resistant strains are active enough to destroy the pathogen, whereas in the susceptible animals small colonies grow rapidly, producing necrotic areas. Macrophages of susceptible mice phago-

cytose large numbers of bacteria, but these bacteria stain and otherwise look normal within the cell. In fact, cells containing thirty to fifty bacteria are suggestive of parasitic reproduction to a point where they may destroy the cell. Bacteria in resistant strain macrophages are difficult to find and, when found, are few in number, are ragged in cell outline, and stain poorly. The picture is one of an intracellular digestive enzyme rapidly destroying the bacteria in the resistant strains, the enzyme being reduced or lacking in the susceptible mice. The differentiation within the macrophages for digestive enzymic ability would explain how the observations of Bordet (181), Rous & Jones (182), and Pike & Mackenzie (183) could be made in a mixed population of phagocytosing hosts and yet have the macrophage system be the important factor in natural resistance.

Bacteria apparently grow rapidly in the susceptible mice, giving rise to a general bacteriemia which usually terminates in death without liver lesions. The large liver lesions of the resistant strains exhibit few or no bacteria, although the effects of highly active toxins are evident. The line demarcating the lesion from normal tissue is composed of dense concentrations of heterophils, macrophages, and fibrocytes. The cells on the normal side are functioning satisfactorily; glycogen and fat are being formed. The physiological functions of the liver are maintained and drawn upon by the rest of the body in the resistant mice. In the susceptible mice, despite little apparent liver damage, glycogen formation has ceased in the whole liver, and since the fat does not stain properly the liver is evidently in dysfunction for the fat as well. This study adds two new characters to those which may be important in differentiating susceptible and resistant individuals in natural disease: the intracellular activity of the macrophages in destroying the bacteria, and the protoplasmic resistance of tissue cells to toxic bacterial products.

Among the other characters which have been suggested as differentiating susceptible from resistant animals is the body temperature maintained by individuals in health and disease. Class differences of this nature in birds and mammals are familiar with respect to tuberculosis, anthrax, etc. Carpenter *et al.* (184) indicated that differences as relatively slight as those induced by artificial fever would destroy the gonococcus. In the animal body a rise in temperature increases phagocytosis. These facts were made the

basis of experiments by Scholes & Hutt (185) on fowl. Their results indicated a higher temperature in resistant than in susceptible birds. Bell (186) finds normal resistant birds with a slightly higher temperature. The reverse is true in birds infected with *S. gallinarum*, the susceptible chicks displaying the higher temperature and the greater death rate. Severens, Roberts & Card (119) confirm Bell's observation on normal chicks and give a series of reasons why they do not consider body temperature important to resistance or susceptibility.

Genetic Basis for Active Immunization

The enhancing of resistance due to a previous contact with a disease may come through infection or vaccination. By comparing six strains of mice genetically differentiated for natural resistance, three lines of heat killed *S. typhimurium* genetically different in virulence, and three periods of vaccination and different dosages, Gowen (107, 187) was able to show that all of these factors were important to acquired resistance. The level of resistance was increased over that which was natural to the strain by nearly one hundredfold as judged by the number of live bacteria necessary to produce disease of equal severity. The position of the genetically different strains with regard to each other did not change in resistance in the vaccinated as contrasted with the unvaccinated strains. This suggests that the genetic factors which make for natural resistance are also important in acquired resistance attained by contact with the disease organism. In a limited sample Gorer & Schütze (113) obtained results suggesting strain differences, but the differences were not significant in the ranges of the numbers of animals employed.

This summary of researches on natural and acquired resistance to disease indicates the complexity of the problem and how completely and along what devious paths the genetic constitution of the host enters into its pathological syndromes.

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COMPLEMENT

By E. E. Ecker[1]

Institute of Pathology, Western Reserve University, Cleveland, Ohio

Introduction

Since the publication of Osborn's *Complement or Alexin* in 1937, the field has been systematically surveyed by Ecker & Pillemer (2), Pillemer (3), Ecker, Seifter & Dozois (4), and finally by Doerr (5).

Notable progress has been made in the past decade. During this time complement has been removed entirely from the realm of the mythical and shown to be a definite entity associated with certain serum globulins. Human and guinea pig complements have been compared, and complement has been shown to function also as an opsonin. With greatly improved methods of assay it has become possible to determine the activity of complement and its components with higher degrees of accuracy.

The following is intended to give a broad appraisal of these newer studies as well as a survey of the trend of these investigations.

Composition and Activity

Available evidence [Ecker, Pillemer & Seifter (6)] indicates that, not unlike guinea pig complement, human complement is composed of four components, the main difference being that, whereas in the guinea pig fraction C′3 is associated with fraction C′1, and C′4 with C′2, in the human both C′3 and C′4 are in persistent association with C′1 and C′2. Ecker & Seifter (7) and Bier, Leyton, Mayer & Heidelberger (8) have also shown that under proper conditions of concentration, all of the corresponding components of man and guinea pig are mutually substitutive. Cushing (9) found that some components can be successfully interchanged among the carp, frog, and guinea pig. The frog's complement is similar to that of the guinea pig, consisting of four components, whereas the carp's complement has at least three components similar to those of the guinea pig and frog. It was not possible to "split" the heat-labile fractions by carbon dioxide. To Cushing, complement appears to have a specificity that is often disregarded,

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i.e., the complement of a given species, or even individual, may react differently with different kinds of antigen-antibody complexes.

Human complement was characterized by Pillemer *et al.* (10) as an euglobulin with an electrophoretic mobility of 2.9×10^{-5} in veronal buffer of pH 7.8 and an ionic strength of 0.1. Examined in the Svedberg oil-turbine ultracentrifuge, the C'1 was found to contain a major component which had a sedimentation constant of 6.9 Svedbergs. It constituted about 0.6 to 0.8 per cent of the total serum protein. Cohn and co-workers (11) have obtained human C'1 in their Fraction III-2. C'1 is inactive per se in both hemolytic and bactericidal systems but will react in combination with the three other fractions.

Human C'1 was further subjected to chemical analysis by Ecker and associates (12). This fraction contained 4.26 per cent polysaccharides and 23.4 per cent hydroxyamino acids.

C'2 was also obtained in a highly purified fraction by precipitation of human serum at pH 6.8 with 2.4 to 2.6 *M* $(NH_4)_2SO_4$ [Seifter, Dozois & Ecker (13)]. It was found in this laboratory that, although it has been difficult to remove all the albumin from this fraction, a globulin was obtained having an electrophoretic mobility of 2.6×10^{-5} in veronal buffer of pH 7.8 and an ionic strength of 0.1. This globulin contained about 2 per cent carbohydrate. Cohn and co-workers (11) found the C'2 fraction in their Fraction IV. Seifter, Pillemer & Ecker (14) observed that this fraction in 0.9 per cent sodium chloride, buffered to pH 6.6, could be preserved for a period of about two years at $-35°C$. C'2 by itself was inactive but fully active when in combination with the other three components.

Except for the fact that C'3 is one of the minimum and relatively labile components of the complex, little is known about this fraction.

Human C'4 was also prepared in this laboratory relatively free from the other components. It had activity of 90 per cent per unit. Although it contained very small amounts of C'1 and C'3 the latter components were readily removed by zymosan. C'4 is highly sensitive to dilute bases and amino compounds.

Bactericidal Activity

Dozois, Seifter & Ecker (15) showed that the killing of *Vibrio comma* by fresh human serum is caused by the combined action of

antibody and complement and that destruction of any one of the four components leads to a loss of bactericidal effect. Evidence was also obtained pointing to the identity of the bactericidal and hemolytic complements. Additional proof of this identity was obtained by the use of purified C'1 and C'2 components [Seifter, Dozois & Ecker (16)].

Fixation

Dozois, Seifter & Ecker (15) found that in bacterial complement fixation inactivation occurs by virtue of the removal of one or more of the components. C'2 and C'3 were the components principally bound. Components fixed to sensitized *V. comma* from specifically inactivated complements retained their power to participate in bactericidal activity.

Thermal Deterioration

Bergenhem (17) observed that, at 39°C., human complement lost practically all its activity in a period of ten hours, and that, in "heat stabilization," C'3 was the component destroyed. Pohl & Rutstein (18) reported that the amount of normal human serum required to produce 50 per cent hemolysis of a standardized sheep red cell suspension is 0.0049 ml. This median is not affected by age, sex, or a forty-eight hour delay of titration at 6°C. At this temperature, however, the median is reduced beyond the lower limits of the normal range in the course of eleven to thirteen days The median decrease in this time interval is 44 per cent. At 23° to 25°C. a similar reduction occurs in two days.

On the basis of kinetic studies made by Wehmeyer (19), it appears that inactivation at temperatures below 40°C. proceeds by a different mechanism from that which occurs between 50° to 56°C.

Effect of Various Agents

According to Gordon & Walker (20), inactivation of guinea pig complement by dyestuffs (congo red series) is not associated with any important chemical changes in the constitution of the compounds. Adsorption or loose union occurs and the effect appears to be dependent more on the molecular size than on any specific grouping. Fibers of cotton and silk reverse the action.

Ready inactivation is induced by processes which affect the state of serum proteins [Boursnell, Francis & Wormall (21)]. Small

amounts of mustard gas rapidly inactivate complement, affecting all the components, and especially C'1 and C'2. Divinyl sulphone inactivates complement even more rapidly. $\beta\beta'$-dichlorodiethylsulphone operates more slowly. Further work is needed to discover whether the effect in this case is due to an oxidation of the SH groups by the β, β'-dichlorodiethylsulphone. The coupling of proteins with the sulfones mentioned produces derivatives with altered immunologic specificity.

Yenson (22) claimed that complement is slightly, but distinctly, inhibited by 0.01 *N* NaF. He also found that, although phosphatase of guinea pig serum is destroyed at 56°C, it is not adsorbed by an antigen-antibody complex as is complement.

In an interesting study on the effects of Mg^{++} and other cations, Mayer *et al.* (23) reported evidence indicating that Mg^{++}, Ca^{++}, Ni^{++}, or Co^{++} is essential for the hemolytic action of complement and that the enhancing action of tissue fluids can also be ascribed to Mg^{++}. Maltaner & Almeida (24) have presented data suggesting that this effect is not only on the hemolytic but also on the clotting activities of serum. In fixation tests for syphilis, however, the enhancing effect of Mg^{++} was neutralized by the inactivated human test serum. Failure to take account of this effect for Mg^{++}-treated complement may lead to errors in the evaluation of titer.

Fixation of Complement to the Antigen-Antibody Complex

Although Haurowitz (25) failed to demonstrate an increase in weight of an antigen-antibody complex in the presence of complement, Heidelberger & Mayer (26) showed that the complex contains more nitrogen than the same complex formed in salt solution, or one formed in the presence of inactivated serum. Heidelberger (27) stated further that, if one accepts 0.05 mg. or 50 μg. as the average amount of complement nitrogen in 1 ml. of guinea pig serum, about 0.2 μg. of complement nitrogen is needed to complete the lysis of about 250,000,000 red cells. Calculated on the basis of complement globulin this represents 1.3 μg. Assuming 150,000 to be the molecular weight of serum globulin, this would represent 6×10^{12} molecules or 25,000 per red cell. Haurowitz & Yenson (25a) calculated that hemolysis of a single red cell required approximately 10^{-11} gm., i.e., more than 10^{10} molecules of saponin or of sodium oleate. The same effect is produced by 1.5×10^{-14} gm., i.e.,

60,000 molecules of complement globulin. The efficiency of complement supports, according to these workers, the enzyme theory of complement action. Heidelberger (27) calculated the number of hemolysin molecules bound as 500 per red cell, a number very small with respect to the number of molecules of complement required. To these authors, the function of complement does not appear to be enzymic in nature. The kinetics of the action of complement on sensitized erythrocytes has been discussed briefly by Mayer & Croft (28), who obtained evidence showing that the antibody resembles an enzyme, whereas complement operates as a cofactor which is used up in the process. Provisionally, Heidelberger & Mayer (26) designated the added nitrogen as C'1 nitrogen, but these authors, in collaboration with Bier (29), as well as Pillemer and co-workers (30), showed that the added nitrogen may be derived from other complement components, C'3 not contributing nitrogen.

Assay

Because of the difficulties involved in the routine determination of nitrogen for complement assay, this method has not found wide application. Several improvements have been made in the method of hemolytic assay. Among these are the employment of spectrophotometry, and the application of the von Krogh equation (31). Mayer, Eaton & Heidelberger (32), and Kent, Bukantz & Rein (33), as well as Ecker, Hiatt & Barr (34) have proposed methods embodying these improvements, the von Krogh equation being used to assist in the accurate interpolation of the values for the 50 per cent hemolytic unit. The theoretical aspects of this work have been discussed by Rice (35) and by Thompson (36).

Mayer *et al.* (37) recently applied similar principles to the quantitative evaluation of complement fixation. In a forthcoming paper, Ecker & Hiatt will evaluate the essential factors to be taken into consideration in the performance of quantitative complement fixation.

In an interesting study of human and guinea pig complements and their components, Bier and co-workers (38) have proposed methods for controlling the adequacy of the reagent for each component. C'3 was shown to be the factor limiting the activity of guinea pig complement, but C'2 was found to be the controlling component in human complement.

Anticomplementary Effects

Jordan (39) claims that there is no direct connection between the complementary power of a given serum and its anticomplementary action. Highly anticomplementary γ-globulins were separated electrophoretically from normal human sera by Davis and associates (40). Purified syphilitic antibody, prepared by dissociation of the specific precipitate, proved to be anticomplementary in a similar fashion. Anticomplementary action was decreased by heating at 56°C. for thirty minutes and abolished by the addition of approximately equal amounts of albumin or β-globulin. It is, however, known that some sera develop the anticomplementary power only after heating. Olhagen (41), in an extensive study on anticomplementary effects, remarked that, in heating strong anticomplementary sera, an opalescence occurred which was not observed in the case of weakly anticomplementary or normal sera. The dilution of such sera prior to inactivation diminished their inhibitory powers, especially when the concentration of electrolytes (buffers) was increased. Filtration prior to heating reduced the inhibitory quality. Olhagen obtained electrophoretic patterns for nine strongly anticomplementary sera, which revealed in two cases the existence of an extra component "X" which migrated between the β- and γ-globulins. In seven cases an abnormal γ-component was observed which was termed "Y_x." It showed higher mobility and lower boundary spreading than a normal γ-globulin. In forty-eight sera exhibiting weakly anticomplementary powers, the abnormal component was not observed. In these cases, however, an increase of the γ-globulin and a reduction of the relative concentrations of albumin was noted. No correlation was found between the anticomplementary power and the amount of γ-globulin found. Hence, a qualitative change of the γ-globulin was assumed to take place. Both by electrophoresis and by chemical fractionation, it was possible to isolate the relatively thermostabile anticomplementary globulin. The separated fraction exhibited the same mobility as the X and Y_x components. It appeared to be a pseudoglobulin with a molecular weight of 170,000 which is considered higher than that of the normal γ-globulin. The Y_x showed two components, one of which had a higher mobility than the unheated globulin. It is believed that this fraction was responsible for the opalescence noted. Olhagen believes the anticomplementary effect is due to a form of denaturation of the

globulin involving a change in dispersion, which leads to complement adsorption. Of 404 cases of liver disease and allied conditions, 18.8 per cent exhibited anticomplementary effects. One third of these sera originated from cases of cirrhosis and chronic or relapsing hepatitis. On the basis of this study, Olhagen recommends that the occurrence of anticomplementary power in a heated serum be given clinical consideration.

In connection with this work, it may be of interest to mention the study of Erickson *et al.* (42). They found the reactive bodies in both syphilitic sera and in biologic false positive sera to be exclusively associated with the γ-globulin, the faster moving "half" of the γ-globulin being serologically more active per mg. of protein than either the whole or the slow moving "half."

An interesting observation by Tyler (43) is the fact that in complement fixation tests attempted with sea urchin fertilizin-antifertilizin mixtures, the fertilizin was found to be highly anticomplementary. There was no evidence of complement fixation with antifertilizin from the species sperm. Instead, a new phenomenon, which was termed "Complement Release," was noted. Either complement was liberated by the bound antifertilizin, or else the anticomplementary action of the fertilizin was neutralized by the antifertilizin. Tyler reported that only C'4 was involved and that the results may imply a chemical similarity between C'4 and antifertilizin. Antifertilizin, however, could not be substituted for C'4 in a hemolytic system.

Complement in Disease

Although abundant evidence exists of the variability of complement in disease, Osborn (1) concluded that

> very much work is required before the clinical significance of complement estimations can be assessed, and it will require much investigation, which some might regard as of an academic nature, before this section of immunological chemistry can obtain any wide application in the diagnosis, prognosis, or treatment of disease.

Ecker and co-workers (44), in a survey of complement in infectious disease in man, arrived at the conclusion that many of the apparent contraindications and inconsistencies encountered in the literature can be attributed to the lack of a uniform technique of complement assay and to an inadequate definition of the limits of "normal" and "abnormal" complement levels.

In general, while the observed complement level often changes in the course of an infection, the extent and direction of the changes are by no means always similar in different diseases, or even in individual instances of the same disease. Furthermore, little information exists concerning the variability of the complement components in the course of infections. In those cases showing a decrease of complementary power, a marked decrease or even disappearance of C'4 occurred. In other cases, in addition to a diminution of C'4, a decrease of C'2 and C'1 was observed. C'3, however, except in one instance, did not show a loss in activity.

In all cases showing no hemolytic complement, the C'4 was strikingly reduced. Interestingly, in normal complement the latter component is one of maximum titer. In the cases studied, C'1 varied only slightly. Frequently, low complement activity was observed in epidemic (meningococcal) meningitis. Nineteen of thirty-eight patients with this disease exhibited diminished complement. In cases of erysipelas, high complement levels were noted.

In a subsequent study, Seifter & Ecker (45) identified complement components in the precipitated urinary proteins of patients with the nephrotic syndrome, and in cases of acute and chronic glomerulonephritis, C'2 was consistently found in the urine. C'3 and C'4 were also detected, but C'1 was seldom found present. As a possible explanation it was pointed out that C'2 is a highly soluble protein, whereas C'1 is an euglobulin. The excretion or retention of the components might therefore be determined by their solubilities. Since antibodies (isohemagglutinins) have also been shown to occur in urinary proteins, it was thought that the excretion of other immune bodies may also occur. The predisposition to infection often shown by patients with kidney disease may possibly be explained on the basis of a loss of antibodies as well as complement.

Both complement and antibody are necessary for the opsonification and destruction of infectants. Ecker & Lopez-Castro (46) have shown that the human components C'1, C'2, and C'4 operate as an opsonin against *Micrococcus candidus*. They have also pointed out that the lability of the human serum opsonin to temperature, aging, action of chemical agents, and adsorbents parallels the lability of its complement. The removal or inactivation of C'1, C'2, or C'4 results in a loss of opsonic activity concomitant with a loss of hemolytic activity. The addition of the

missing component to the inactivated serum or fraction thereof leads to a recovery of both opsonic and hemolytic qualities. Maaløe (47) also independently concluded that "after thorough qualitative and quantitative comparisons between alexin and opsonin, nothing has been found that does not agree with the identity-theory." Maaløe therefore pleads for the introduction of a single term for the "normal, thermolabile antibacterial factor in serum," i.e., a new common term for complement and opsonin. Since the term "alexin" implies "protective substance" its use to designate opsonic complement is suggested by the present author. The term "complement" can then be reserved for the hemolytic, bacteriolytic, and bactericidal complements.

Employing a more accurate method of assay similar to those used by Mayer, Eaton & Heidelberger (48), and by Kent, Bukantz & Rein (49), Ecker, Hiatt & Barr (50) observed that in pregnant guinea pigs substantially lower complement activities occur than in nonpregnant animals and that in the postpartum period there is a gradual rise in complement activity approaching a level normal for nonpregnant females. The results, treated statistically, showed that the value of "t" in the tests of significance were well above the minimum values required to demonstrate significant differences among the groups.

Although it has long been known that a reduction in complement activity occurs during malarial paroxysms [Cathoire (51), Vincent (52), Wendlberger & Volavsek (53), Zermati & Vargues (54)], further work, employing more accurate methods, was necessary to establish the loss in comparison with the complement activities of healthy individuals. Dulaney (67) stated that the 50 per cent unit of hemolytic complement for thirty individuals ranged from 0.0032 ml. to 0.006 ml., with a median of 0.0045 ml., and that day-to-day variations in these individuals appeared to be slight. No marked variation from the normal was noted in the sera of twenty-five patients with liver disease, or in thirty-two patients with noninfectious diseases of various types. However, in 178 titrations of the sera of twenty-four neurosyphilitics treated with malaria, complement was usually diminished. This decrease in some instances was slight, but in severe cases was great. Two of the patients showed reductions from 0.0048 ml. before inoculation to 0.08 ml. on the tenth day after inoculation, and 0.28 ml. on the twentieth day after inoculation. One of these cases had a preinfec-

tion titer of 0.0039 ml. and on the thirteenth day following inoculation a titer of 0.0134 ml. On the twenty-first day the titer stood at 0.225 ml. Two patients showed moderate decreases, and two showed slight changes. Interesting indeed is the observation that the complement depression coincided with, or followed, the peak of parasitemia for that individual. Dulaney was, however, unable to correlate the complement values with the number of the parasites. Huddleson (68) reported that, in brucellosis, a rapid and complete termination of the disease can be accomplished either in the experimentally infected guinea pig or in the human being by making use of the potentiating property of certain of the sulfonamides on the serum antibody-complement system. If fresh serum from the infected animal or man tested during the oral administration of the drug (sulfadiazine or sulfamerizine) shows a bactericidal effect against the organisms in the test tube, and if this action persists *in vivo* for at least six days, then all the organisms in the body fluids will be destroyed, and recovery will ensue. On the other hand, if the disease has progressed to the point where a large portion of Brucella serum antibodies have apparently lost their power to bind complement (Neisser-Wechsberg phenomenon), the presence of the sulfadiazine in the blood in high concentration (30 mg. per cent) has no appreciable effect on the antibodies. The solution of this problem was accomplished by the combined use (in transfusion) of normal whole blood, plasma, or serum, and the oral administration of small quantities of the drugs.

According to Spink & Hall (55) the killing action of normal human blood for Brucella does not require the presence of leucocytes. The effective antibody is heat-stable, and complement appears to be necessary for its optimum bactericidal action. *Brucella abortus* is more readily destroyed than *B. suis* or *B. melitensis.*

Jordan (39), in his studies on complement in disease, went so far as to state that "if in a patient with icterus the complementary power of the serum is found high, this is an argument . . . against the existence of an atrophic cirrhosis." In some cases of Weil's disease, and in other patients with "catarrhal jaundice," normal or even high complement levels were observed. However, the anticomplementary power of the sera of patients with metastatic tumors in the liver was not consistently either high or low. Strikingly high values were found in patients with mechanical obstruction of the bile ducts. Also, low values were obtained in patients

with diseases of the bones (Kahler's, Schüller-Christian's, Albers-Schönberg's diseases and carcinomatosis). Local affections of the skeleton (tuberculosis, tumor), however, resulted in no changes. In lipoid nephrosis low values were observed.

Of nine dogs with alloxan diabetes [Candela & Urgoiti (56)], seven showed a diminution of complement varying between 12 and 68 per cent. The drop in level appeared after twelve hours, and in the majority of the animals the glucemia increased after twenty-four hours or later. Extirpation of the pancreas [Horster (57), Bayer & Form (58)] was found to lead to a diminution of complement activity. No knowledge exists as to the components involved.

In a recent summary, de Gara & Goldberg (59) reported the complement levels in 107 children (age range three days to fifteen years) who were healthy at the time of the test and during a preceding four-week period. Titers ranging from 0.0040 to 0.0069 ml. were found in 83.5 per cent, with higher titers in 6.3 per cent and lower in 10.2 per cent. The mean titer was 0.0053 ± 0.00115 ml. There was no relationship between complement activity and age, sex, or season of the year. Low complement activities were observed in 20 per cent of children who were sick at the time of the test, having upper respiratory infections, pneumonia, meningitis, rheumatic fever, nephritis, or other diseases.

Bieler, Spies & Ecker (60) noted no significant deviations from the normal in the sera of eight persons with hypoproteinemia without infections.

Miscellaneous

Complement and leucocyte.—The role of complement in the physiology of the leucocyte has been investigated by Delaunay & Pagès (61). Chemotaxis appeared to be impossible in the absence of complement, and phagocytosis was enhanced by its presence. This observation introduces another link in the relationship between complement as an opsonin and the phagocyte [Ecker *et al.* (46), Maaløe (47)].

Hemoflagellates.—Chang & Negherbon (62), in a study of the hemoflagellates, claim that inactivated antisera retain their lytic power and that prolonged storage of sera does not significantly destroy the lytic power, although the agglutinative power may be reduced. Formolized, phenolized, or heat-killed organisms are lysed more rapidly in the antisera than are the living cells.

Leptospiras and Treponemata.—Stavitsky (69), of this laboratory, has also noted that lysis of *Leptospira icterohemorrhagiae* occurred in the presence of specific antibodies and heat inactivated normal sera. In other words, it might be assumed that heat inactivation does not necessarily destroy all the components of complement. It appears to the present author that considerable confusion in the literature has resulted from the assumption that heat inactivation leads to complete destruction of all four components, which is actually not the case.

Complement appears to be necessary for the demonstration of protective antibodies in the sera of syphilitic rabbits [Turner (63)].

Typhoid bacilli.—When sensitized typhoid bacilli are cultivated in the presence of complement, it was found by Morris (64) that they may grow in one preparation of complement and fail to grow in another. He interpreted this observation as showing that the residual infectivity of a mixture of the bacilli with their homologous antibody is not due to the presence of unaltered bacilli, but rather to bacilli which, after exposure to the antiserum, no longer behave as normal organisms.

Viruses.—Concerning the still obscure question of the role of complement in virus neutralization, Morgan (65) points out that the loss of neutralizing capacity for the virus of Western equine encephalomyelitis occurring in antisera upon storage at 4°C., could be fully restored by the addition of complement. She states further,

> If the analogy with the bactericidal effect of complement+antiserum under favorable conditions would hold, then virus-neutralizing antibody+complement may perhaps be virucidal.

Since complement fixation tests are today in greater use than ever before, it seems appropriate to call attention to the work of Wertman & Plotz (66). They found antibodies for typhus antigens in commercial frozen and dried complement apparently resulting from the use of sera of guinea pigs employed in the testing of epidemic typhus vaccines.

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THE NATURE OF ANTIBODIES

By Dan H. Campbell[1]

The Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California[2]

As more precise physical tools and methods are developed and applied to the chemistry of proteins, the biological as well as physical nature of antibodies become clearer and our concepts less controversial. Some of these concepts are now based on such substantial evidence that they must be accepted while others represent varying but logical interpretations of indirect evidence. This review gives briefly the general immunochemical properties of antibodies and the attendant current interpretations as to their physical and biological significance. A more detailed discussion of antibodies in the recent literature will be found in Boyd's book (1) and a review chapter by Campbell & Lanni (2).

Physical Properties

Chemical composition.—Very little study has been made of the chemical composition of antibody protein, due primarily to the lack of precise chemical analytical methods and sufficient quantities of purified antibody proteins. Smith, Green & Bartner (3) recently studied the amino acid and carbohydrates composition of plasma and colostrum globulins of cattle, as well as plasma globulins of humans and horses. Although they found some slight differences in composition, the immunochemical significance is questionable since their preparations were chemical fractions of plasma and probably contained only small amounts of antibody protein. The older work which has been discussed by Boyd (1) and Marrack (4) indicates that serum globulins are fundamentally similar, and if differences do exist between antibody of different species or different antibodies in the same species more quantitative analytical methods will be required to detect any significant chemical differences. In fact, antibodies can only be characterized chemically in a general manner as typical serum globulins which may occur either as euglobulins or pseudoglobulins or both.

[1] Owing to unforeseen circumstances Dr. Pauling was unable to contribute his portion of the chapter as planned. However, the author is indebted to him for criticism and help with this manuscript.

[2] Contribution No. 1200.

Electrokinetic properties.—It is now generally recognized that antibodies have a fairly characteristic electrokinetic charge which places them in the gamma globulin component or intimately associated with it. An exception to this is contained in a report by Seibert & Nelson (5), who found that antituberculin in rabbits migrated with the α-globulins. The characteristics electrophoretic charge apparently bears no relation to solubility properties or molecular weight and suggests a common chemical constitution.

Molecular weight.—The development of techniques for the determination of molecular weights of large molecules has advanced to a point where we can now be reasonably sure of the values. Although three methods are available, namely, osmotic pressure, centrifugation and light scattering (6), the use of sedimentation constants from ultracentrifugation has contributed the greatest amount of data. From the mass of data available, there emerges the interesting fact that antibodies occur in two classes, namely, the so-called low molecular weight class of about one hundred and sixty thousand and the high molecular weight class of about one million. [An excellent report on ultracentrifugal studies of proteins is given by Pedersen (7).] Both types may occur in the same animal, as, for example, the horse, which produces a predominance of high molecular weight antibody in response to injection of pneumococcus and low molecular weight antibody in response to diphtheria toxin. This brings up the possibility of relationship between the nature of the antigen and the molecular weight of the antibody. A recent report by Deutsch *et al.* (8) indicates that some of the antibodies in human serum, particularly those responsible for blood types, may also occur in the high molecular weight fraction.

Immunochemical Properties

Nature of the antibody combining site.—Very little is known about the actual structure of the antibody combining site, but it is generally assumed to be a pocket-like structure on the surface of the globulin molecule having a three dimensional configuration related to that of some small portion of the antigen which stimulated its formation and furnished the specific pattern. This idea arises from the extensive studies made by Landsteiner and others (9), who have established our present basic concepts of serological specificity.

One might expect that the functional activity of an antibody molecule would be reflected in its specificity as an antigen, i.e., that the specific configuration of the antibody combining site would act as an antigenic determinant; however, this has never been demonstrated. For example, the quantitative studies by Treffers & Heidelberger (10) and Treffers, Moore & Heidelberger (11) failed to show any antigenic difference between normal and antibody globulin. One of us (44) also failed to obtain any cross reactions between horse anti-Type I pneumococcal globulin and rabbit anti-Type I pneumococcal globulin using anaphylaxis tests in guinea pigs. The failure of the antibody combining site to confer a specific antigenicity is of considerable importance since it may have bearing on the nature of the combining site. There are several explanations not presently susceptible to experiment that might be proposed. For example, the site may be highly liable to enzymatic digestion so that it does not survive long enough to afford an antigenic template for antibody formation. Again, our idea of a pocket-like structure representing the combining site may be correct, but such a structure represents only a small proportion of the total molecular surface and may thus afford a pattern for antibody formation which is insignificant with respect to the template activity of the rest of the molecule. From Landsteiner's studies (9) it has become evident that the size of the antibody combining site approximates that of a phenyl arsonic acid group which would represent approximately 1.0 per cent of the surface of a prolate ellipsoid the size of an antibody molecule of 160,000 mol. wt. Although Haurowitz (12) concluded that only one azo arsanilate group per protein molecule was sufficient to induce the formation of antiarsanilate antibodies, the statistical significance of such values is doubtful.

Stability of the antibody combining site.—In view of the author's interest in the possibility of producing artificial antibodies by denaturation and renaturation of proteins in the presence of a suitable template (antigen), considerable interest has been given to the problem of the stability of the antibody combining site. Since this subject has been reviewed by Campbell & Lanni (2) and limited space does not permit further elaboration here, it is enough to say that the combining site has been found to be relatively stable to the ordinary denaturing agents such as heat, alcohol and urea and that much of the apparent destruction of antibody ac-

tivity is often due to the formation of complexes with nonantibody protein which results in covering the antibody combining site. Even spread films at water-air interfaces have been found to be active (13), and such results have been used by Neurath *et al.* (14) to conclude that Pauling's theory of antibody formation and structure (15) is impossible. Such conclusions are of course unjustified until more knowledge of the intimate structure of such films is available.

The number of combining sites.—The exact number of combining sites or valences which occur on antibody molecules is still debatable, and, in fact, this number may vary to a considerable degree in any given antiserum. The bulk of the evidence at present seems to indicate that the usual type of antibody resulting from active immunization studied in connection with precipitation reactions is predominately bivalent. The ultracentrifugal studies of Pappenheimer, Lundgren & Williams (16) indicated that horse antitoxin molecules are capable of combining with two molecules of toxin, and the surface chemistry studies of Harkins, Fourt & Fourt (17) showed that alternate layers of antibody and antigen films could be deposited on slides. Our own studies (18) on the composition of precipitates of antibodies and polyhaptenic simple substances also suggested the bivalence of precipitating antibody. Evidence for the necessity of at least two combining sites for the formation of a precipitating framework was presented in the studies of Pauling *et al.* (19, 20). Thus, a dye antigen containing one arsanilic acid group and one carboxylic acid group would not precipitate with either antibody alone but would when added to a mixture containing antibodies against phenyl arsonate and phenyl carboxylate. Abramson *et al.* (21) also concluded that agglutinating antibodies must contain two or three combining sites. Although it seems reasonable that multivalence of antibodies would be required for precipitation or agglutination such a condition might not be necessary for other types of antigen-antibody reactions such as those of hypersensitivity or lysis. From a theoretical standpoint the possibility of masked valences becomes of great speculative interest. Thus the possibility arises that the reactive valences which are present on the molecular surface of an antibody molecule are only a portion of the total number and that the globulin molecule represents a compact formation of smaller units, some of which might have an antibody configuration. Hints of such pos-

sibilities are found in reports on studies of partially denatured antibodies where an increase in combining capacity was produced. For example, Tyler (22) found that photodynamic irradiation of a variety of rabbit antiserums resulted in a loss of precipitating or agglutining power, but such antibodies would still attach themselves to their respective antigens and inhibit reactions between antigen and complete antibodies. The titers of some inhibiting serums were three to four times greater than would be expected upon the basis of activity before the denaturation. Such an idea of hidden valences would be incompatible with Rothen's observations (23) and conclusions that antibody forces are operative over large distances since one would expect that any internal valences would still be operative.

The question of valence also brings up the problem of whether a given antibody molecule can have combining sites of more than a single specificity. Studies by Heidelberger (24) and Haurowitz (25) and more recently by Lanni & Campbell (26) have consistently failed to demonstrate any such "heteroligating" antibodies. These studies have involved antibodies against two separate antigens injected simultaneously as well as antibodies against two or more determinant groups on the same antigen molecule.

The heterogeneity of combining sites.—There seems to be little doubt that antisera contain antibody molecules showing a great variation in reaction capacity. Conceivably this heterogeneity can arise from several sources. Perhaps the most obvious arises from the foregoing discussion of valence in which the number of combining sites will vary. This has been suggested in several reports in which antibodies were found which would combine with antigens, as evidenced by subsequent inhibition reaction, but which showed only weak or no precipitation reaction. Pappenheimer (27) and Heidelberger *et al.* (28) found that during the early stages of immunization of a horse with egg albumin, the antibodies were detectable chiefly by their inhibiting effect on egg albumin when good precipitating antibodies were subsequently added to the system. Miller & Campbell (29), in studies of serum from persons allergic to ovalbumin, found that these serums contained antibodies (reagins) which attached to ovalbumin and became incorporated in the precipitate formed upon the addition of a good precipitating antiovalbumin serum from rabbits. There is also considerable evidence that the so-called Rh blocking antibodies are

univalent since they stick tenaciously to Rh positive cells but produce no agglutination. However, the univalence of such antibodies is still not definitely established and the problem requires much more investigation.

A second type of heterogeneity might be expected to result from the possibility that different portions of the antigen molecule would be involved in supplying the pattern for antibody formation. Although the resulting antiserum would contain antibodies only for a given type of antigen molecule, there would be a great variation between individual antibody molecules with respect to the portion of the antigen molecule toward which the combining site was directed. Thus a whole spectrum of antibodies might be expected to be produced around a single group and consequently against a single antigen molecule.

A third type of heterogeneity would be expected to arise with respect to variations in the degree of complementariness.

There is of course ample evidence that heterogeneity does occur but the factors involved are still not clear. Goodner & Horsfall (30) found that considerable variation occurred in the protective power of antibodies obtained by serological fractionation of horse and rabbit antipneumococcus serums with specific polysaccharides. The quantitative aspects of heterogeneity have been studied by Pauling, Pressman & Grossberg (31), and Haurowitz, Cindi & Schwerin (32) have described some so-called intermediates between antibodies and normal serum globulins.

The nature of forces involved in antigen-antibody reactions.—Pauling *et al.* (33) have concluded that the forces responsible for combination and attraction of antigens and antibody molecules may be classified as electronic van der Waals attraction, Coulomb attraction, attraction of electric dipoles or multipoles, and formation of hydrogen bonds. The spatial complementariness of the specific combining sites of the antigen and antibody permits close contact of considerable surface, allowing these weak forces to cooperate over fairly large areas and thus to form a strong antigen-antibody bond. Such forces and their significance have been generally accepted without question for many years, but recently Rothen (23) has interpreted the results of experiments on unimolecular film studies of antigen-antibody reactions as indicative of so-called "long range forces" which are operative over relatively long distances. These experiments consisted essentially of placing a

layer of antigen on a polished metal slide and covering it with "barriers" of various thickness and then exposing the barrier surface to antibody. Specific antibody combination was detected when barriers of barium stearate, octadecylamine, protein multilayers or polyvinyl formal resin (Formvar) showing average thicknesses of 100 to 200Å were interposed between antigen and antibody. If the results are not due to artifacts resulting from structural discontinuities of the barriers it is obvious that an entire new concept has been given not only for serological reactions but many other fundamental biological mechanisms. The possibility of discontinuities in barrier structure is very real and must be ruled out before definite conclusions are made. Certain indirect evidence introduces some question as to long range forces operative in antibody reactions. Thus, O antibodies fail to react with a cell coated with Vi antigen, and antibodies against the hemoglobin of red blood cells cannot react when the cell membrane is intact. Similarly, proteins covered by haptens lose their native specificity. Although the thickness of the Vi layer is not known it seems reasonable to expect that it would be small. The thickness of the membrane of the red blood cell has been estimated by Waugh & Schmitt (34) and Zwickau (35) to be about 200 Å.

The site of antibody formation.—The site of antibody formation still remains a problem but due to the work of White & Dougherty (36) and Harris & Ehrich (37) interest has shifted from the reticuloendothelial system to lymphocytes and plasma cells. The former authors found that when adrenal-cortical hormone was used to produce a temporary lymphopenia in immunized rabbits a concomitant rise in antibody titer occurred. This is thought to be a direct result of dissolution of lymphocytes with liberation of globulin. The latter authors feel that both the reticuloendothelial and lymphatic systems play an important role in antibody formation but that the phagocytic cells take up particulate antigens, such as bacteria, and partially digest them into soluble antigenic fragments which are then released and taken up by the lymphocytes. This would of course imply that the actual antibody-forming mechanism was in the lymphocytes or plasma cells. It is interesting to note that serum proteins in general have been thought to originate in the liver [Madden & Whipple (38)], and hence the question immediately arises as to whether there are other sites of serum protein formation and whether such special-

ized proteins as antibody globulins arise *de novo* from amino acids or are actually modified globulins.

The formation and structure of antibodies.—Any discussion of the formation and structure of antibodies must still be highly speculative, but the experiments reported by Pauling & Campbell (39) and partially confirmed by Freidrich-Freksa (40) indicate that the specific affinity of a protein for a dye can be increased by denaturation and renaturation of a protein in the presence of the dye. These findings partially support the earlier ideas (15) regarding the structural nature of the antibody combining site.

The question of course arises as to what constitutes an antibody, and although a precipitating antibody from rabbits may afford a convenient model we have seen in the foregoing discussion of heterogeneity that many types of antibodies are possible. Experiments have been carried out during the past several years by Pauling and the author in an attempt to produce protective antibodies against pneumococcus by renaturing proteins in the presence of capsular polysaccharide. These trials have been mostly negative although a few preparations have given slight specific protection (44).

Recent experiments with C^{14} labeled leucine by Kooyman & Campbell (41, 42) have shown that antibody globulin can enter into the so-called "dynamic equilibrium state of the body" without loss of antibody properties. This finding is in contradistinction to the conclusions reached by Heidelberger *et al.* (43) working with N^{15} labeled glycine. The C^{14} experiments were performed by injecting the labeled DL-leucine into actively immune rabbits and into passively immunized rabbits. The passively transferred antibody as well as the actively formed antibody showed considerable C^{14} activity in a relatively short time. Horse antibodies injected into rabbits failed to take on any C^{14} activity. Whether this indicates that a heterologous protein cannot enter into exchange reactions or whether the failure is due to the large molecular (horse anti-SII and SI) structure is not known at present.

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PATHOGENIC STREPTOCOCCI

By Charles H. Rammelkamp and John H. Dingle
Department of Preventive Medicine, School of Medicine
Western Reserve University, Cleveland, Ohio

Introduction

Investigations dealing with streptococci producing disease in man and animals have been so numerous during the past decade that a review of the "pathogenic" streptococci must necessarily be limited. Primary consideration will therefore be given here to the streptococci that are responsible for disease in man.

All streptococci may be "pathogenic" for man if circumstances are favorable. It has become increasingly clear, however, that the β-hemolytic streptococci of Lancefield's Group A have a greater pathogenicity for human beings than do the organisms of other groups. The Group A streptococci have accordingly received the most attention from bacteriologists concerned with the processes of infection. This review will consider some of the recent studies on the β-hemolytic streptococci under the following headings: Growth and Isolation, Serological Groups and Types, Streptolysins, Streptococcal Leucocidin, Fibrinolysin, Streptococcal Proteinase, Hyaluronic Acid and Hyaluronidase, and the Erythrogenic Toxin. Insofar as possible the cellular composition and products of these organisms will be considered in relation to the disease process and immunity.

Growth and Isolation

It has long been known that pathogenic streptococci grow best on enriched media and that their nutritional requirements are complex. Hutner's review (1) in 1938 emphasized the limited knowledge of the factors essential for growth of hemolytic streptococci at that time. The problem has since been studied by a number of investigators and considerable progress has been made (2 to 8). The nutrition of enterococci has been reviewed recently by Niven & Sherman (9). Media of known composition have been devised (10 to 14), and a satisfactory medium has been developed for the determination of resistance of Group A streptococci to sulfonamides (15).

Studies of the role of streptococci in human infections have

been hampered by the lack of an entirely satisfactory method for isolation and preliminary identification of the organisms. It is common experience that the more frequently cultures are obtained in a given population, the higher will be the pharyngeal carrier rates for the various Lancefield groups of streptococci (16). While this result is undoubtedly a function of the method of swabbing and the numbers of streptococci in the pharynx, it is also a function of the kind of medium and type of blood employed. In this case, as with other bacteria, the use of a selective medium is advantageous.

Selective media.—The greatest advance in developing a selective medium for isolating hemolytic streptococci from the upper respiratory tract was made by Pike (17). The medium contains tryptose, glucose, and rabbit blood in a total volume of 2 ml. Just prior to use, 0.15 ml. of an autoclaved aqueous solution of sodium azide (1:1000) and 0.1 ml. of an autoclaved aqueous solution of crystal violet (1:25,000) are added to each tube. Swabs obtained from the tonsils and oropharynx are placed directly in this medium and incubated for approximately eighteen hours, following which subcultures are made on blood agar plates. The selective medium permits the growth of hemolytic streptococci and inhibits staphylococci, *Neisseria*, *Hemophilus*, and coliform organisms (17, 18).

In a preliminary study of one hundred thirty-nine cultures, Pike isolated hemolytic streptococci from only sixteen by direct plating, whereas fifty-one isolations were made by use of the selective medium. Direct plating of a throat swab may fail to show hemolytic streptococci unless several thousand organisms are present (18).

Pike's method for culturing hemolytic streptococci has given results that alter considerably the current concepts of the frequency of the carrier state. In a study of nine hundred throat cultures from 756 children, serologically identified streptococci were isolated from 42 per cent (19). With this method also, repeated throat cultures from the same individual increased the likelihood of isolating hemolytic streptococci.

Other selective media have been used for the isolation of streptococci. Potassium tellurite enrichment broth appears to have no advantage over the sodium azide and crystal violet medium of Pike (20). Potassium tellurite medium has been used to permit the selective growth of nonhemolytic streptococci (21, 22), and media

containing sodium azide have been employed for the inhibition of gram negative bacteria (22, 23, 24).

Media containing blood.—The usual practice in isolating β-hemolytic streptococci is either to streak the cultures directly on blood agar plates or to make subcultures on blood agar after preliminary growth in a selective medium. Many different types of blood have been employed for this purpose and controversy has arisen concerning the constancy of the reaction of streptococci on blood from different species (25, 26). Early workers believed the hemolytic reaction to be relatively constant (27). Cumming (25), however, found marked differences in the behavior of eighteen strains isolated from sputum. All of them were hemolytic on human blood agar, but eleven of them produced only a greenish discoloration without lysis of the cells on rabbit blood agar. He termed these strains "pseudohemolytic" and noted further that failure to hemolyze rabbit blood was likely to occur after several subcultures. Of the eleven "pseudohemolytic" strains, six produced no hemolysis of guinea pig blood and three failed to hemolyze mouse blood.

Fry (28) studied six strains of β-hemolytic streptococci which produced a greenish zone but no β-hemolysis when grown aerobically on horse blood agar. Dance & Murray (29) observed the behavior of streptococci from human sources on human, horse, beef, sheep, and rabbit bloods and concluded that human blood was the least affected. Two strains produced a green color on blood from three different human donors but gave β-hemolysis on horse blood. Traut & Johnson (30) studied eighteen strains, one of which hemolyzed human but not sheep blood while two lysed sheep but not human blood.

Dingle *et al.* (31) examined 181 strains of β-hemolytic streptococci after several weeks' storage in a frozen state. All had originally given β-hemolysis on a medium containing horse blood. Serological identification of all the strains was not carried out. The strains were subcultured on media containing horse, sheep, and rabbit bloods, with the results shown in Table I.

In a study of the type of blood most suitable for isolation of β-hemolytic streptococci, Pike (32) examined 199 throat swabs which were first cultured in a selective medium and then streaked on human and rabbit blood agar plates. Hemolytic streptococci were isolated from 44 per cent of the human blood plates and from

33 per cent of the rabbit blood plates. Only one strain lysed rabbit cells and failed to hemolyze human cells. Sheep blood has been recommended for purposes of isolation because it eliminates the problem of differentiating β-hemolytic streptococci from *Hemophilus hemolyticus* (33). Human blood has a similar though less marked effect.

TABLE I

TYPE OF HEMOLYSIS PRODUCED BY STRAINS OF β-HEMOLYTIC STREPTOCOCCI AFTER STORAGE

Source of blood used in medium	Strains showing indicated type of hemolysis				Strains failing to grow	Total no. of strains
	β	α′	α	None		
Horse	115	33	2	0	31	181
Sheep	79	67	7	0	28	181
Rabbit	79	62	7	4	29	181

The occurrence of nonhemolytic variants of β-hemolytic streptococci also presents a problem (25). Griffith [cited by Colebrook (34)] has described two such variants. Todd (35, 36) and Lancefield (37) have observed the development of nonhemolytic variants in strains passed through mice. A nonhemolytic variant of a Type 12 strain of streptococci has been reported as the cause of an epidemic among infants (38). Colebrook *et al.* (34) reported an outbreak of Type 12 streptococcal infections among the patients on a plastic surgical ward. The organisms from the first cases produced β-hemolysis on blood agar plates (39), but later, as new infections continued to occur, eleven strains of streptococci producing neither α- nor β-hemolysis on horse blood agar were isolated. Subcultures of these strains did not hemolyze human or rabbit blood. The eleven strains were all Group A, Type 12 organisms. When a meat infusion and peptone nutrient agar base was used with horse blood, the strains produced α- or greenish hemolysis. Two hemolytic variants, however, were obtained from these strains. During the same study, two other strains were isolated which failed to produce hemolysis on blood agar plates; one was a Type 11 streptococcus, the other a Type 25. More recently, Isaacs (40) has produced viridans variants by growing streptococci of Groups A, C, E, G,

and L under increased oxygen tension. These organisms, however, lost their serological group characteristics.

The above observations indicate that both the type of blood and biological variation in the organisms may at times make difficult the recognition of hemolytic streptococci. Other factors must also be considered. Fry (28) states that Llewelyn Jones pointed out a number of years ago that β-hemolysis may be more readily demonstrated on streaked plates if a stab is made into the agar while inoculating the plate. Brown (27) observed that deep colonies may be readily identified, and Rantz & Jewell (41) and Brown (42) were able to correlate the type of hemolysis with the serological group in poured blood agar plates.

The poured blood agar plate has not been widely used because of technical difficulties. The modification of Perry & Petran (43), where the plate is first streaked and blood agar subsequently poured on as a second layer, simplifies the procedure to some extent. Poured plates or streaked-poured plates have been compared with the streaked plate in three recent studies (31, 32, 44). In two of these studies (31, 32) the poured plate or streaked-poured plate showed no distinct advantage over the streaked plate. Rantz (44) concluded that skillfully prepared streaked plates were satisfactory.

It has been recognized by several observers (28) that certain strains of streptococci exhibit β-hemolysis when cultured anaerobically but fail to do so aerobically. These results have recently been clarified by Herbert & Todd (45). In a study of the hemolysis produced by thirty-two strains of Group A streptococci, these investigators found two strains which produced streptolysin S but not streptolysin O. These two strains caused hemolysis when grown either on the surface or deep in the blood agar plate (45, 46). When the strains isolated by Colebrook *et al.* (34) were examined for streptolysin production, it was found that they produced only streptolysin O. These strains failed to exhibit hemolysis when streaked on the surface of blood agar plates and incubated either aerobically or anaerobically. In poured blood agar plates, however, the same strains cause good hemolysis. It was concluded that the hemolysis induced by surface colonies was due to streptolysin S, the streptolysin O being oxidized. At the lower oxygen tension of deep colonies, both streptolysin O and S are responsible for hemolysis. In the complete absence of oxygen, however, strep-

tolysin O is inactive. It seems apparent, therefore, that strains producing only streptolysin O cannot be detected by routine surface streaking of blood agar plates.

The presence of high titers of antistreptolysin in blood employed for making the media may also inhibit hemolysis produced by hemolytic streptoccoi. The blood of certain horses, for example, may contain large amounts of antistreptolysin O.

There is no information concerning the freqency with which Group A strains producing only streptolysin O are encountered. The not infrequent failure to isolate β-hemolytic streptococci from certain patients with scarlet fever (47) may be an indication of infection with such strains. Obviously, more information is required before the relative merits of the type of blood employed and the use of streaked, streaked-poured, or poured agar plates can be determined.

Serological Groups and Types

It is now well recognized that the β-hemolytic streptococci may be classified into groups and types by serological methods (48). Group specificity is dependent upon a polysaccharide, commonly known as the C substance, which may be removed from the organism by Lancefield's acid extraction (49) or Fuller's formamide method (50). The polysaccharide is apparently an integral part of the cell, is nontoxic, and is not related to immunity in animals or man (48). The precipitin technique is used to determine the group. Thus far twelve groups of streptococci have been recognized. Many studies (48, 51 to 54) have established the fact that Group A organisms are responsible for the majority of infections in man. It is probable, however, that organisms of all the groups may be pathogenic when circumstances are favorable (48).

Various studies of the antigenic composition of Group A streptococci have shown that two substances, designated M and T, are responsible for type-specificity (55). The M antigen is an alcohol-soluble protein which may be extracted from the cell by heating at a pH of 2 to 3 (56, 57). The antigen is presumed to lie at or near the surface of the cell since enzymatic digestion removes it without killing the streptococcus (58). The M substance is now considered to be closely associated with virulence. Strains lacking the M antigen are avirulent (59). Immunization with killed virulent or-

ganisms confers type-specific immunity (55, 57, 60, 61) and anti-M sera protect mice from infection with the homologous strain. Watson, Rothbard & Swift (62) have produced type-specific immunity in monkeys by intranasal inoculation and have demonstrated that such immunity is correlated with the development of the antibodies to the M antigen. These investigators have pointed out that immunity in man to hemolytic streptococcal infections is probably type-specific since in no known instances have recurrent nasopharyngeal infections been due to the same type of streptococcus. Recently Jersild (63) reported an instance of second infection with the same type of streptococcus in a patient who had previously received early and intensive therapy with penicillin for scarlet fever. It is possible that early therapy in the patient interfered with the development of type-specific immunity. Type-specific M antibodies have been demonstrated following natural infections in man (64, 65).

The second antigen responsible for type-specificity in Group A streptococci is the T substance which has been defined by the recent work of Lancefield & Dole (66). The M protein was first destroyed by pepsin or trypsin; further digestion resulted in liberation of the T substance from the cell. Following additional purification, the substance was found to be a protein, free from nucleic acid, with an isoelectric point of pH 4.5. The soluble form of the T substance was antigenic, but the antibodies so produced did not protect the animal against infection with an organism containing the same T antigen.

Typing of Group A β-hemolytic streptococci may be accomplished by either the agglutination (67) or precipitin (68) technique. The agglutination technique, as described by Griffith (67), is dependent on the T antigen and to a lesser extent on the M antigen, whereas the precipitin reaction is dependent on the M antigen alone. Studies on a large number of strains of hemolytic streptococci by Griffith's technique of typing have shown that cross-reactions are common due to the presence of related T antigens in various types of Group A organisms (69, 70). For example, streptococci of Types 15, 17, 19, 23, and 30 possess similar T but distinct M antigens, and cross-reactions among these types are frequently found by the agglutination technique (70). Although most Group A streptococci possess both M and T antigens, Lancefield & Stewart (71) found twenty-eight strains belonging to

Types 14, 17, 19, 24, 26, 28, 29, and 30 which apparently lacked the T antigen. Streptococci with serologically distinct T and identical M antigens are rare. Watson & Lancefield (72) have shown that Types 10 and 12 possess similar M antigens.

Until relatively recently most epidemiological studies have been based on data obtained by the Griffith technique of typing. Technical and interpretive difficulties have been common, as Elliott (73) and Hilles & Hamburger (74) have emphasized. In one study of 333 group A strains (74), 165 failed to type or gave cross-reactions which rendered definitive identification impossible by the agglutination method. These 165 strains were later typed by the capillary precipitin technique (68). The advantages of the precipitin technique include: (*a*) the rapid formation of the precipitate; (*b*) elimination of difficulties due to granularity of the cultures; (*c*) identification of the large number of strains which exhibit cross-reactions or fail to react in the agglutination method; and (*d*) the relationship of the M antigen to type-specific immunity.

The M-precipitin technique, as generally carried out, however, may not alone be the answer to the problem of typing. Elliott (75) has studied fifty-eight strains of Group A streptococci which could not be typed when grown at 37°C. Thirty of these strains could be typed when grown at 22°C. It was found that at 37°C. these strains produced a proteinase which destroyed the M substance. At the lower temperature this enzyme was not produced. M substance is usually found in Group A streptococci isolated from patients with infections, but glossy variants are devoid of this antigen, although they may retain the T substance. Recently Rothbard & Watson (76) found that 42 per cent of the strains isolated in the convalescent stages of infection exhibited a progressive loss of M substance and in three instances this antigen disappeared entirely. No loss of the T substance was observed. These observations suggest that the typing of carrier strains in particular should include an analysis of both the M and T antigens.

Streptolysins

Streptococcal hemolysins have been studied extensively since 1895 when Marmorek (77) demonstrated that fluid cultures of these organisms lysed red blood cells. Early it was thought that streptococcal hemolysin was produced only in culture media containing serum and that the hemolysin so obtained was extremely

labile and was nonantigenic. In 1926 Neill & Mallory (78) reported the production of a hemolysin by streptococci in a serum-free medium. The hemolysin lost its activity in the presence of oxygen but could be reactivated by certain reducing agents. It was stable if stored in the absence of oxygen. These conflicting results were partially clarified by the work of Todd (79), who showed that streptococci produced at least two different hemolysins, called streptolysin O and streptolysin S, and that both streptolysins were present in preparations such as Weld's hemotoxin (80). It is now known that streptolysin O is sensitive to oxygen but may be stored in the reduced state. Streptolysin S, which is obtained by extracting streptococcal cells with serum (80), is extremely sensitive to heat and cannot be reactivated by reducing agents.

In 1932 Todd (81) showed that streptolysin O was antigenic and that the antibody produced by this hemolysin did not neutralize streptolysin S. The biochemical properties of a highly purified preparation of streptolysin O have been determined (82, 83). It was found to be a heat-labile protein with S-S linkages. The purified preparation did not hemolyze red cells unless activated by cysteine, glutathione, thiolacetic acid, and sodium thiosulfate. In 1933 Todd (84), and later Abdalla & McLeod (85), reported that serum-streptolysin was antigenic, but that the antibodies which were produced did not neutralize serum-streptolysin itself, but rather streptolysin O. It seems probable that their preparations contained both streptolysins since it was later demonstrated that streptolysin S was antigenic only when the animal was immunized with living cultures (86). Later Herbert & Todd (87) were able to demonstrate conclusively that streptolysin O and S are distinct hemolysins. Most Group A strains produce both streptolysins but occasionally a strain is encounteerd that produces no streptolysin O. Using several of the latter strains, Herbert & Todd (87) were able to show that the hemolysin was not stable and that inactivation was irreversible. Dried streptolysin S is relatively stable. Partially purified streptolysin S contains tyrosine, argenine, and tryptophane. It is mainly protein, but a low nitrogen and high phosphorous content suggests the presence of some other constituent. Evidence has been obtained that streptolysin S is, or is associated with, a lipoprotein.

Because streptolysin S is unstable, the demonstration of anti-

streptolysin S is a difficult laboratory procedure. In contrast, antistreptolysin O titers are relatively easily measured and serological determination of these antibodies has, therefore, been employed in the study of infections caused by streptococci of Groups A, C, and G. In the past, some difficulty has been experienced in the production and storage of streptolysin O for use in antistreptolysin determinations. Rantz & Randall (88) have obviated this difficulty by the storage of a concentrated preparation which is activated with the cysteine just prior to use. Reliable methods for determining the antistreptolysin content of patients' sera are needed. Ipsen (89) has described an accurate and simple method for calculation of the antistreptolysin titer, based on the use of standard human sera containing known amounts of antistreptolysin. Ipsen has offered to supply reference sera to investigators interested in this problem. Another method has been described by Löfgren (90), who also emphasized that contamination of sera with *Pseudomonas fluorescens*, *Pseudomonas pyocyanea*, and *Bacillus subtilis* leads to false elevation of the antistreptolysin titer.

In contrast to fibrinolysin, no quantitative method for measuring streptolysin O production by β-hemolytic streptococci has been described (91). There may be some variation in the capacity of specific types of Group A hemolytic streptococci to produce this hemolysin. In one study (92) Type 3 β-hemolytic streptococci appeared to stimulate a greater antibody response, following infection in man, than did Type 19. Similar observations have been reported by others (91).

Streptococcal Leucocidin

In the past, streptococcal leucocidin has been thought to be a specific substance which acts on leucocytes. Using newer techniques Todd (93) has demonstrated that streptolysin O has a powerful lytic action on leucocytes when tested at low oxygen tension. The lysis of leucocytes proceeded more slowly than did the lysis of red blood cells under similar conditions. Attempts to produce a leucocidin free from streptolysin O from Group A streptococci have failed. Streptolysin S had no effect on leucocytes. No correlation could be demonstrated between virulence of the organisms and their capacity to produce leucocidin *in vitro*. Todd concludes that there is no evidence of any streptococcal leucocidin apart from streptolysin O.

Fibrinolysin

In 1933 Tillett & Garner (94) observed the lysis of human fibrin clots by culture and culture filtrate of β-hemolytic streptococci. Later, Milstone (95) showed that purified human fibrinogen was not affected by streptococcal fibrinolysin, but that an accessory substance, termed lytic factor, was required. This substance, or lytic factor, was found to be associated with the euglobulin fraction of human serum and exhibited alone no activity on human fibrin. It occurred to Kaplan (96) and others (97 to 102) that Milstone's lytic factor might be similar to the lytic agent termed "globulin substance" (103). The latter substance was also associated with the euglobulin component of plasma and was rendered fibrinolytic by treatment with chloroform. When so activated it is a tryptic enzyme capable of digesting various substrates including fibrin (104).

Kaplan (96) found that both lytic factor and globulin substance were activated by organic solvents to give a proteolytic enzyme and that fibrinolysin activated the inert precursor, lytic factor. Independently, Christensen (98) demonstrated that lytic factor was activated by streptococcal fibrinolysin.

Fibrinolysin has been partially purified (98) but has not been characterized chemically. The activated enzyme, although proteolytic, is not trypsin (99, 105). Antiprotease can inhibit the activity of the enzyme (100, 106, 107). It is of some interest that activated fibrinolysin destroys prothrombin (108).

A new terminology for the various reacting agents has been suggested (99). Streptokinase is substituted for fibrinolysin, plasminogen for lytic factor or globulin substance, and plasmin for the activated enzyme. The inhibitory substances are then termed antistreptokinase and antiplasmin.

For a number of years the resistance of plasma clots to streptococcal fibrinolysis has been used as the basis for serological tests in the study of streptococcal infections, and the development of such resistance has been observed in the blood of patients during the period of convalescence (109). That such resistance might not be specific for streptococcal infections was suggested by the fact that plasma clots obtained from the new-born (110, 111, 112), from various animal species (94), and from patients with a variety of other diseases (110, 113, 114) also exhibited resistance. This apparent "nonspecific" resistance to streptococcal fibrinolysis has

been explained by the observations of Kaplan (107). Theoretically, resistance of plasma to fibrinolysis may be caused by: (*a*) the presence of specific antifibrinolysin; (*b*) the presence of antiprotease (antiplasmin) or antiplasminogen; and (*c*) a deficiency of lytic factor or plasminogen. Antifibrinolysin combines only with fibrinolysin and has no effect on the activated enzyme, plasmin, nor apparently, on its precursor, plasminogen. An incubation period of approximately thirty minutes at 37°C. is required for neutralization of most of the fibrinolysin by its specific antibody. The two substances combine in multiple portions. Antiplasmin inhibits streptococcal fibrinolysin by inhibiting the active enzyme plasmin. The high antiplasmin content of sera obtained from patients during the acute phase of febrile diseases, as well as that of the sera of various animals, is probably responsible for the resistance of their plasma clots to streptococcal fibrinolysis (107, 115). There is evidence that antiplasmin is associated with the albumin fraction of serum (100). No evidence has been obtained for the existence of an antiplasminogen.

Much of the early work on fibrinolysin and antifibrinolysin had to be carried out by methods that were essentially qualitative. Recently a quantitative method for measuring these two substances has been reported (116, 117, 118). Using this method it has been shown that fibrinolysin is produced by streptococci of Groups A, C, and G, in confirmation of the observations of Wu (119). Occasional strains of streptococci of Groups B and F produced fibrinolysin in small amounts (117). In one study the median titers of fibrinolysin produced by streptococci of Groups A, C, and G were as follows: Group A, 117; Group C, 61; and Group G, 20. It would thus appear that strains of Group A streptococci are better able to produce fibrinolysin than strains of other groups. There is evidence, though still incomplete, that fibrinolysin production by strains of Group A streptococci may be related to the serological type. Thus, Type 3 organisms usually produce very small quantities of this substance, whereas Types 12 and 14 usually produce large amounts. It is of interest that organisms which produce proteinase (75) fail to produce fibrinolysin (120). The production of fibrinolysin may be relatively constant for a given strain of Group A streptococcus. This point was investigated during an outbreak of exudative tonsillitis and pharyngitis due to a Type 5

hemolytic streptococcus. Organisms isolated from the throats of patients at the onset of the disease, and six weeks later, produced similar quantities of this substance. Treatment with sulfadiazine had no effect on this characteristic (121).

The development of a quantitative test has greatly increased the value of antifibrinolysin determinations in the serological diagnosis of streptococcal disease. In a population of young male adults it was found that 88 per cent of individuals who harbored no β-hemolytic streptococci in their throats exhibited titers of 150 or less in their sera (122). Sera having a high antifibrinolysin titer usually showed an elevated antistreptolysin titer, suggesting that antifibrinolysin was produced as the result of a streptococcal infection. The reverse, however, was not true. Many sera showed elevated antistreptolysin titers with normal antifibrinolysin levels, indicating that many streptococcal infections are not followed by an elevation of the fibrinolysin antibodies. This interpretation was confirmed in a study of sera of one hundred and fifty-one patients with streptococcal pharyngitis. All of the patients had an increase in the antistreptolysin titer of their sera during convalescence. Only 37 per cent of the patients showed a rise in antifibrinolysin titers. These results, as well as those of others (123, 124), demonstrate that the determination of the antistreptolysin titer in acute and convalescent sera is a more sensitive diagnostic test than the determination of antifibrinolysin levels.

There is a discrepancy in the frequency of antifibrinolysin responses following streptococcal infection as determined by the quantitative antifibrinolysin test (122) and the plasma-clot resistance test (109, 125). Results with the plasma-clot resistance test have in general yielded an incidence of antifibrinolysin responses greater than 37 per cent. A possible explanation is that the development of antifibrinolysin following infection is related to the ability of the infecting organisms to produce fibrinolysin (96, 116, 117, 126).

It has been suggested that fibrinolysins vary immunologically (127), since it was found that the resistance of plasma clots varied with fibrinolysins produced by different strains. This hypothesis has not been confirmed, however, and the available evidence indicates that fibrinolysins produced by streptococci of Groups A, C, and G are immunologically identical (54, 128, 129).

Proteinase

The production of a muscle-digesting enzyme, histase, by some strains of hemolytic streptococci has been reported by Frobisher (130), and subsequently by Seegal & Seegal (131). In 1945 Elliott (75) found that Group A streptococci produced an extracellular proteolytic enzyme, termed streptococcal proteinase. This enzyme digests the M antigens of streptococci, fibrinolysin or streptokinase, fibrin, casein, and milk. Streptococcal proteinase is active only under reducing conditions and an inactive precursor of the proteinase may be produced when the organisms are grown in certain media (132). Rothbard & Todd (133) found that Group A streptococci, isolated from patients during the acute and convalescent phases of infection, produced remarkably constant amounts of proteinase. Production of the enzyme does not appear to be related to the serological type of the infecting organisms or to the clinical characteristics of the disease process (133), although further evidence is required to establish these observations. The enzyme is antigenic in the horse. Patients with Group A streptococcal infections, however, fail to develop high antiprotease titers during convalescence (134). Further study of this enzyme and its role in the infectious process is required.

Hyaluronic Acid and Hyaluronidase

In 1937 Kendall *et al.* (135) first identified hyaluronic acid, a mucopolysaccharide, as a constituent of the capsular material in Group A streptococci. Shortly thereafter Seastone (136) demonstrated a similar substance in the capsules of Group C streptococci of animal origin. In 1940, Meyer *et al.* found hyaluronidase in the supernates of cultures of hemolytic streptococci (137). Since that time students of streptococcal infections have been concerned with the hyaluronic acid-hyaluronidase system because it may throw light on the pathogenesis of streptococcal infections (138). Several reviews concerning hyaluronic acid, hyaluronidase, and spreading factors in general have recently been published (139, 140).

The relationship of hyaluronic acid to encapsulation has been the subject of considerable study. Hyaluronic acid has been found in mucoid or matt strains (141, 142). Although it is present only in encapsulated strains, all streptococci with capsules do not contain this mucopolysaccharide. Strains of encapsulated streptococci

lacking hyaluronic acid have been found in groups A, B, C, G, and M (141, 143, 144). In a study of hemolytic streptococci isolated from human sources, Seastone (141) found that seventy-five of of eighty-one Group A strains produced enough hyaluronic acid in the culture medium to be detected in dilutions of 1:10 or greater. All of the strains producing large amounts were encapsulated. None of four strains belonging to B and C produced detectable amounts of hyaluronic acid. No relationship between the hyaluronic acid content of the capsule and the type of Group A streptococcus has been described.

The possible relation of hyaluronic acid to virulence of streptococci was indicated by the early work of Seastone (136), who observed that highly invasive strains of Group C streptococci isolated from guinea pigs produced more mucopolysaccharide than nonpathogenic strains. The capsules of Groups A and C streptococci disappeared almost immediately upon the addition of testicular (143) and leech (145) extracts, as well as of hyaluronidase prepared from Group C organisms (143). A similar effect was observed *in vivo* in the peritoneal cavity of the mouse using leech extract (145). The capsule of Group B organisms was not affected (143).

If hyaluronic acid is a major factor in the virulence of streptococci, it might be expected that hyaluronidase would exert a protective effect in experimental infections. Hirst (145) found that mice infected with ten thousand minimum lethal doses of Group C encapsulated streptococci could be completely protected by treatment with leech extract. Similar treatment of mice infected with a Group A, Type 3 streptococcus, however, showed only slight evidence of protection. It was concluded that the virulence of Group C infections in mice is related to the hyaluronic acid content of the capsule. McClean & Hale (146), however, failed to protect mice against either Group A or Group C streptococcal infections by the use of testicular extract. This result was thought to be due to the inhibitory effect of serum on the activity of the enzyme. Other investigators have since reported that Group A and Group C infections in mice could be altered favorably by testicular extracts, while heat-inactivated hyaluronidase failed to exert a beneficial effect (147, 148). It would thus appear that the hyaluronic acid content of the capsule of streptococci may be an important factor with respect to animal virulence, but further clarification is needed.

With respect to virulence for human beings, the early investigations (141, 149, 150, 151) indicated that strains of Group A streptococci isolated from severe infections produced more hyaluronic acid than those isolated from moderate or mild infections, suggesting that virulence for human beings might also be related to hyaluronic acid production. *In vitro* studies (148) have demonstrated that streptococci of Group A treated with hyaluronidase were more susceptible to phagocytosis by human leucocytes than organisms not so treated. In the study of this problem, as well as an investigation of methods, Pike (152) examined 229 strains of Group A streptococci isolated from well carriers and 54 strains isolated from patients with a variety of infections. Sixty-four per cent of the carrier strains and 48 per cent of the strains from infections produced detectable amounts of mucopolysaccharide *in vitro*. Unless the sources of these two collections of strains are biased through selection, these figures indicate that carrier strains are more likely to contain hyaluronic acid than are strains from infected patients. Pike further observed that the quantitative determination of mucopolysaccharide was a more reliable index of hyaluronic acid production than was the demonstration of capsule formation or the morphology of the colonies on various laboratory media. Capsules were demonstrated in approximately 42 per cent of all the Group A strains, which is in close agreement with the figure of 46 per cent that Crowley found in examining 339 strains of Group A streptococci recently isolated from patients with streptococcal infections (153). The available evidence thus fails to indicate any close relation between virulence for man and either encapsulation or hyaluronic acid production.

Following the identification of hyaluronidase in the supernatant fluid of cultures of hemolytic streptococci (137), a great deal of work was directed towards this enzyme because of its capacity to function as a spreading factor (139, 140). Studies were carried out on the conditions favorable to the production of the enzyme by streptococci, on the occurrence and distribution of strains of streptococci producing the enzyme, on the relation of enzyme production and capsule formation, and on the relation of the enzyme to the infectious process.

The production of hyaluronidase by streptococci was found to be enhanced by the addition of the specific substrate, hyaluronate, to the culture medium (143, 154). For example, in a simplified

medium, a Group C and Group A strain of streptococci were found to produce six hundred and sixteen hundred mucin clot-preventing units, respectively (154). On addition of crude hyaluronate to the medium, however, six thousand four hundred and twenty-four thousand mucin clot-preventing units were produced by these two strains. With the Group C streptococcus the enzyme production per unit of weight of bacterial nitrogen was directly proportional to the amount of hyaluronate added to the medium. The capacity of a strain of streptococcus to produce hyaluronidase apparently cannot be enchanced by repeated subculture in a medium containing hyaluronate (144).

The occurrence and distribution of hyaluronidase in β-hemolytic streptococci of the different groups and types have been investigated to only a limited extent. It would appear that hyaluronidase production occurs less frequently in the strains of Group A than in strains of Groups C, G, and L (138, 143, 144, 155, 156). Interpretation of these results is difficult, however, since there may be a marked variation in the potency of the same strain of streptococcus from day to day (138). Moreover, hyaluronate has not been added routinely to the culture medium when testing for hyaluronidase activity.

In the most extensive study of hyaluronidase yet reported, Crowley (153) examined 376 strains of β-hemolytic streptococci from human sources. There were 308 strains of Lancefield's Group A, representing twenty-eight serological types as determined by Griffith's technique (67). Sixty-four of these Group A strains produced hyaluronidase and all of them were either Type 4 or Type 22. Seventy-two per cent of the Type 4 strains and 96 per cent of the Type 22 strains produced the enzyme. Considerable hyaluronidase was produced in some instances, since the supernatant fluids of twenty-eight strains showed activity in a dilution of 1:1000. The sixteen Type 4 strains and the one Type 22 strain which initially failed to produce hyaluronidase were retested with the same results. Hyaluronidase was produced by thirty-five of the fifty-five Group C strains and by all of the thirteen Group G strains.

Pike (157) studied the hyaluronidase production of 110 nonencapsulated Group A streptococci in a medium containing added hyaluronic acid. Fifty-five per cent of the strains reduced the hyaluronic acid content of the medium within twenty-four hours. Additional strains exhibited apparent hyaluronidase activity when the

incubation period was prolonged to several days. No information concerning the types of streptococci examined was presented.

There are conflicting reports regarding the relation between hyaluronidase production and capsule formation by streptococci. McClean (143) has stated that hyaluronidase production and capsule formation are mutually exclusive, since the enzyme will prevent the formation of capsules. The hyaluronidase-producing strains examined by Crowley (153) were not encapsulated. Pradhan (158) studied a Group C streptococcus that exhibited spreading factor only in filtrates of young cultures which contained encapsulated organisms. Spreading factor has been observed in preparations from streptococci which contained no measurable amount of hyaluronidase (156). Other investigators (159, 160), however, have found a good correlation between diffusion factor and hyaluronidase. Since spreading occurs with substances other than hyaluronidase, these observations are difficult to interpret (139), although it is possible that variations in the methods used to measure the enzyme activity may be in part responsible. Substances exhibiting hyaluronidase activity always produce spreading, but spreading factors do not always contain hyaluronidase. It is common experience, however, that capsules appear in young cultures and disappear as the culture ages. In one study of three encapsulated strains (143), two were found to produce a trace of hyaluronidase after twenty hours of incubation. The capsules had disappeared from the organisms at the time the hyaluronidase activity was demonstrated. Pike (157, 161) noted that some encapsulated strains of Group A streptococci destroyed hyaluronic acid present in the medium. In general, hyaluronidase activity in such strains is weak in comparison with the nonencapsulated streptococci, but it would appear that some organisms may be able to produce both hyaluronic acid and hyaluronidase. It has been emphasized by Meyer (138) that failure to demonstrate hyaluronidase does not necessarily indicate the absence of this enzyme. Further studies are obviously indicated.

The possible relationship of hyaluronidase activity to infectious processes has been discussed exhaustively elsewhere (139, 140). In regard to streptococci, McClean's experiments are of interest (144). Nonencapsulated, hyaluronidase-producing strains of Groups A and C streptococci exhibited low virulence when injected intraperitoneally in mice, whereas encapsulated strains var-

ied greatly in virulence. On intracutaneous inoculation of mice, however, some of the hyaluronidase-producing strains exhibited greater variance than the capsulated strains. In rabbits, encapsulated organisms were more apt to produce localized lesions in the skin, while hyaluronidase-producing strains were associated with spreading.

Little correlation has been found between hyaluronidase production and virulence for man (153). Of 127 strains isolated from cases of scarlet fever, only thirty-five or 27.6 per cent showed hyaluronidase activity; similarly only fifty or 36.8 per cent of 136 strains from cases of pharyngitis showed production of the enzyme. Hyaluronidase-producing organisms have been likewise isolated from healthy individuals.

The inhibition of spreading factor and of streptococcal hyaluronidase by specific antisera has been described (139, 159). Using hyaluronidase prepared from a Group A, Type 4 streptococcus and from Group C streptococci, McClean (159) showed that the antihyaluronidase produced was group-specific but not type-specific. The degree of specific inhibition of the enzyme by antisera may be measured by the mucin clot-prevention test of McClean, Rogers & Williams (162). In a recent study by Friou & Wenner (163), this test was employed in the examination of sera collected from patients with rheumatic fever and scarlet fever and from a control group. Sera from patients with rheumatic fever showed high inhibitory titers for streptococcal hyaluronidase; moderately high titers were found in the sera from patients with scarlet fever in comparison with the sera from normal individuals. These results are difficult to interpret in view of the data reviewed above, which indicate that relatively few types of Group A streptococci produce appreciable amounts of hyaluronidase. The importance of defining the relation of streptococci to rheumatic fever is so great, however, that these observations should be extended.

Erythrogenic Toxin

Although considerable interest was manifested in the erythrogenic toxin immediately after its description by the Dicks in 1924 (164), few studies have been reported in recent years. This is probably due to a number of factors, including the decline in the mortality rate of scarlet fever, the development of effective chemo-

therapeutic agents, the toxic reactions which attend immunization procedures, and finally, the lack of antibacterial immunity following immunization with the toxin.

The erythrogenic toxin has been employed as an immunizing agent (165, 166) and as a test (Dick test) for the study of the epidemiology of scarlet fever (16). Stock (167) has developed a method for concentrating the toxin by absorption on aluminum silicate, elution, ammonium sulfate precipitation, and dialysis. Using this method ten million skin test doses were obtained per milligram of concentrated materials. Later Stock (168, 169) prepared a heat-coagulable protein containing two hundred million skin test doses per milligram and described its physical properties. The concentrate has been used for immunization and is effective (166). Veldee has also described methods for partial purification of the toxin. Since the toxin is not stable, he has suggested the use of human serum as a stabilizing agent (170, 171).

In an excellent review of the present status of the effectiveness of erythrogenic toxin as an immunizing agent, Toomey (172) points out that in various contagious disease hospitals, from 8.6 to 17.7 per cent of nurses with a positive Dick reaction develop scarlet fever, whereas only 0.6 per cent of immunized nurses acquire this disease. Various authors have raised the question as to what this reduction in the scarlet fever attack rate actually indicates. Both an increase and a decrease in the incidence of tonsillitis without rash has been reported in immunized subjects (173 to 176). Further clinical studies are necessary, but the weight of evidence appears to indicate that immunization with erythrogenic toxin affords little or no specific antibacterial immunity. The work of Strumia (177) has demonstrated that immunization with erythrogenic toxin does not stimulate the production of antifibrinolysin, antistreptolysin, or streptococcal agglutinins. Since immunity to streptococcal infections is largely type-specific (48), it is difficult to understand how protection against streptococcal disease can be afforded by immunization with the toxin.

Until relatively recently it has been thought that the erythrogenic toxin produced by various strains of Group A β-hemolytic streptococci was a single immunological substance. Hooker (178) has pointed out, however, that numerous observations do not conform with this view. Joe (179) has observed patients with a negative Dick test who developed scarlet fever, and others who had

antitoxin in the blood during convalescence but whose Dick test remained positive. Mixtures of toxin and antitoxin may appear to be completely neutralized when tested in the skin of one individual, yet are apparently incompletely neutralized when tested in the skin of another person. The administration of antitoxin is not always beneficial in patients with scarlet fever (178, 180) and relapse may occur when a patient sustains a cross-infection in the hospital (181). These discrepancies led Hooker & Follensby (178) to a study of the nature of the erythrogenic toxin. By immunological and chemical criteria, they identified two erythrogenic toxins termed A and B. Most Dick-positive individuals react to the A toxin and not to the B toxin. The existence of immunologically distinct erythrogenic toxins may explain some of the above discrepancies observed in the study of scarlet fever.

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THE SPIROCHETES[1]

By Gordon E. Davis

Rocky Mountain Laboratory, Division of Infectious Diseases
National Institute of Health, Hamilton, Montana

From a broad biologic viewpoint the pathogenic members of the order Spirochaetales have no more claim for discussion than the saprophytic or free-living forms. However, the following discussion pertains, for the most part, to the pathogenic forms with reference to other forms as is deemed necessary.

The most serious attempt to bring some order into the classification of this group of organisms, which have been claimed by both protozoologists and bacteriologists, is given in the recent edition of *Bergey's Manual of Determinative Bacteriology* (1). There is still little agreement as to accepted species, and even some generic designations are not accepted by many workers. This is particularly true of the genus *Borrelia*, as most of the newly described species of blood spirochetes are placed in the genus *Spirochaeta*, which was erected by Ehrenberg in 1833 for *Spirochaeta plicatilis*, which is free-living in fresh or salt water.

As the new classification varies widely from the old, it is presented here in outline. It will be noted that there are two families instead of the former one, and three genera in each family. There are forty accepted species and approximately two hundred "species" listed as inadequately described.

Family I. Spirochaetaceae Swellengrebel 1907
- Genus 1 *Spirochaeta* Ehrenberg 1833
- 2 *Saprospira* Gross 1911
- 3 *Cristispira* Gross 1910

Family II. Treponemataceae Schaudinn 1905
- Genus 1 *Borrelia* Swellengrebel 1907
- 2 *Treponema* Schaudinn 1905
- 3 *Leptospira* Noguchi 1917

The division into families is based primarily on the length of the organisms and secondarily on the presence or absence of protoplasmic structures: axial filaments, protoplast membranes, and cross-striations. The families are further characterized by their biotopes.

[1] This review covers the period up to April, 1948.

Members of the family Spirochaetaceae Swellengrebel 1907 have coarse spirals and are thirty to five hundred microns in length. They may be free-living or parasitic and are found in water and in the intestinal tract of bivalve mollusks.

Members of the family Treponemataceae Schaudinn 1905 have coarse or slender spirals, four to sixteen microns in length, have no obvious protoplasmic structures, and, with few exceptions, are parasitic on vertebrates.

The genus *Spirochaeta* is separated from the genera *Saprospira* and *Cristispira* in that both periplast membrane and cross-striations are absent in the former while they are present in the two latter genera. In turn, the genus *Saprospira* is separated from the genus *Cristispira* in that species of *Saprospira* are free-living in marine ooze while species of *Cristispira* are parasitic on lammelibranch mollusks.

Five species of *Spirochaeta* are listed as adequately described and twelve additional names are listed in the appendix. The type species is *Spirochaeta plicatilis* Ehrenberg. Three species of *Saprospira* are accepted and two additional names are listed in the appendix. The type species is *Saprospira grandis* Gross. Three species of *Cristispira* are also accepted and twenty-three additional species which appear to belong to the genus are listed in the appendix. The type species is *Cristispira balbainii* (Certes).

The family Treponemataceae is divided primarily on the basis of staining reactions and secondarily on conditions of growth. Species of the genus *Borrelia* stain easily with aniline dyes while species of the genera *Treponema* and *Leptospira* stain with difficulty except with Giemsa's stain and silver impregnation. Species of the genus *Treponema* are strict anaerobes while species of the genus *Leptospira* are aerobes.

Species of the genus *Borrelia* are further characterized by a length of from eight to sixteen microns, with coarse, shallow, irregular spirals, generally tapering to a fine filament. They are parasitic and some are pathogenic for man, others for mammals and birds. Some are transmitted by arthropods. The type species is *Borrelia anserina* (Sakharoff). There are seventeen accredited species and a hundred or more additional terms in the appendix. Ten of the accredited species (and ten listed in the appendix) cause relapsing fever in man, one a spirochetosis of fowls and one of mammals. Three are found on mucous membranes, one in the

stomach contents of the tsetse fly and one from lesions in hogs suffering from hog cholera.

Species of the genus *Treponema* are given a range in length of from three to eighteen microns. These species have acute, regular or irregular spirals; terminal filaments may be present. Some species stain only by Giemsa's stain. They are pathogenic for man or other animals and generally produce local lesions. The type species is *Treponema pallidum* (Schaudinn & Hoffmann). There are eight accredited species and thirty-five or more names listed in the appendix. At least three species are pathogenic for man and one for rabbits.

Species of the genus *Leptospira* are defined as finely coiled organisms, six to twenty microns in length. They are seen in living preparations only by dark field and stain with difficulty except with Giemsa's stain and by silver impregnation. They require oxygen for growth. The semicircular hook at one or both ends separates these organisms from all other species. The type species is *Leptospira icterohaemorrhagiae* (Inada & Ido) Noguchi. There are four recognized species and twenty-one additional names listed in the appendix. Three of the four accepted species are pathogenic.

Morphology.—The present classification is based primarily on morphology as observed by the compound microscope. Within the family Spirochaetaceae, the presence or absence of certain protoplasmic structures serves for primary differentiation. Also, within the family Treponemataceae, loose spirals, tightly wound spirals, and curved ends clearly separate the type species of the three genera. However, morphology ceases to be a criterion of species.

In a refreshing study of a free-living species, Dyar (2) reports the isolation in pure culture of an organism believed by him to be *Spirochaeta plicatilis* Ehrenberg. This organism multiplies readily on the red cell fraction of blood. Studies on the structure of this species were made by light- and dark-field illumination, by staining methods, and by the phase and electron microscopes. A cell-membrane, which is supposed to be absent in species of the genus *Spirochaeta*, was demonstrated by use of the author's cell-wall stain. Although the cross-striations, which have been considered characteristic of the *Saprospira* and *Cristispira*, could not be shown by staining methods or by the electron microscope, the phase microscope regularly revealed such structures. It is, therefore, considered that this spirochete is not a single cell organism, but a

multicellular filament. There was no evidence of flagella. Although an "axial filament" was apparently shown following overnight staining in Giemsa's solution, this is considered by the author as an artifact. As a result of the demonstration of a cell-wall and of cross-striation, it is concluded that the validity of *Spirochaeta* and *Saprospira* as distinct genera is in question. Morton & Anderson (3, 4) have studied the morphology of *Leptospira icterohaemorrhagiae*, *L. canicola*, and *Treponema pallidum* by use of the electron microscope. In a preliminary report a "slime sheath" and thin tendrils projecting from the organisms were described. Granules and lateral buds were also reported in the treponemas. Internal structures, such as granules or extruding granules, were not observed in the leptospiras; neither was there any evidence of flagella. Wile, Picard & Kearney (5) recognized a complete and continuous membrane-like sheath around *Treponema pallidum* with flagellar-like processes at various points along the organisms, but not at the end.

Mudd, Polevitzky & Anderson (6) report a similar study of *Treponema pallidum*, *T. microdentium*, and *T. macrodentium*. There was no evidence of an axial filament but a delicate cell-wall or periplast was shown, and flagella, often in groups of four, were found along the sides or near the ends of *T. pallidum* and *T. macrodentium*. Spheroidal dense bodies attached to the spirochetal cell were interpreted as asexual reproductive bodies. Such bodies have been frequently observed by workers with the blood borrelias, and various interpretations placed upon them.

In a study of *Borrelia novyi*, Lofgren & Soule (7) came to the conclusion that no real additional knowledge had been obtained over what had formerly been known.

Baltazard (8) has recently reported that by careful dark-field examination the louse-borne *Borrelia recurrentis* can be distinguished from tick-borne species, the former presenting a uniform refractile aspect, the latter exhibiting a "double contour" appearance.

The Borrelias

World War II afforded considerable opportunity for a study of the methods of control of louse-borne relapsing fever, although efforts were aimed primarily against louse-borne typhus. There was also ample opportunity for extensive observations on the arsenicals and penicillin in treatment. The louse-borne epidemic in

North Africa and in Egypt did not compare in extent with the great epidemics in Poland and Roumania during World War I, nor were they associated with typhus to the same degree. It has been suggested that the origin of the epidemic was in Fezzan, Libya, spreading to Tunisia in 1943, Algeria in 1944, Morocco and Egypt in 1945. In Egypt the epidemic progressed from thirty-three reported cases in January, 1945 to more than five thousand in January, 1946.

Much information on the tick-borne disease was obtained from new areas, in some instances as the result of military activities. Cases appeared for the first time in Cyprus (9) in soldiers who had slept in caves. The vector was found to be *Ornithodoros tholozani.* This same species was incriminated in Cyrenaica (10) for the first time in Africa. Other foci were demonstrated at Tobruk (11), in the Bizerte-Ferryville-Mateur region in Tunis (12), and in Cullinam (13) and Xinavane (14) in southern Africa. *O. verrucosus* was reported as a vector in Azerbaidjan (15) where the disease was recognized for the first time.

A ten-year old record, only recently seen, is of considerable interest. In 1938, Avanessov (16) observed cases in natives and Europeans in Afghanistan. The cases were associated with an undetermined species of *Ornithodoros.* This observation extends the records of tick-borne relapsing fever from the eastern shores of the Mediterranean to the western borders of India. An endemic center in Afghanistan may have some bearing on the occurrence of the disease in India, the origin of which has never been explained.

Relation to arthropod hosts.—The relapsing fever borrelias cannot be discussed apart from their arthropod hosts. Brumpt (17) has postulated that these organisms were originally parasites of arthropods, secondarily adapted to the blood of vertebrates. Perhaps "commensals" would be a more exact term. Nicolle & Anderson (18) advanced the hypothesis that these spirochetes commenced as parasites of small mammals, became adapted to humans through the agency of ticks, and later, through the close association with lice, were able to survive in and be transmitted by these insects. Epidemics could be readily explained if any tick-borne species or strain could be conserved over considerable periods of time in the louse. Several workers have observed the survival of tick-borne strains in the louse over a period of several days but are in agreement that there is no multiplication within the coelo-

mic cavity while *B. recurrentis* multiplies very rapidly. On the other hand, some tick-borne borrelias survive and maintain their full virulence over periods of years even under complete fasting condition of the specific tick host. In some species of *Ornithodoros*, transovarial transmission is sufficient to maintain the strain without recourse to infective blood meals. In other species, the per cent transovarial transmission is very low and there is evidence that in still other species it does not occur. Results obtained in the study of one species cannot safely be applied to another species. A maximum of 100 per cent transovarial transmission has been reported by Davis (19) for *O. turicata* while only 0.29 per cent is reported for *O. hermsi* by Wheeler (20). In an attempt to explain the appearance of infective *O. turicata* in certain areas in Texas, following a relatively long period over which it had been impossible to demonstrate spirochetes in the tick population of a cave, transovarial transmission was dismissed as a factor by Wisseman (21), reference being made to the reported per cent transmission of *O. hermsi* instead of the reported per cent transmission of *O. turicata*, the species under study.

The specific relation which exists between a species of *Borrelia* and its normal tick host has been emphasized by Brumpt (22) and has been studied extensively in the Western Hemisphere by Davis (23) and by Mazzotti (24). There is a 100 per cent specificity between the three species of Borrelias, viz., *B. hermsii*, *B. turicatae*, and *B. parkeri*, found in the western United States and their respective vectors *Ornithodoros hermsi*, *O. turicata*, and *O. parkeri*. Following the recovery of a strain of spirochetes from a patient, it has been possible to determine the tick vector by these specific studies. In two instances such results have been confirmed by finding the determined species of tick in the home of the patient. In laboratory infections, it has been possible to determine the species of the infecting organism by simiar studies. Mazzotti found that *O. amblus* and *O. furcosus* did not transmit *B. turicatae* or *B. venezuelensis*, and that *O. parkeri* and *O. hermsi* did not transmit *B. venezuelensis*. There are numerous exceptions to this degree of specificity, the most recently reported being the transmission of *B. microti* by *O. lahorensis* and *O. canestrinii*, by Delpy, Rafyi & Maghami (25). There are three prime necessities in such studies—known species of Borrelias, known species of *Ornithodoros*, and a "clean" stock of the species of tick under study. Over a period of

years in specificity studies, the reviewer has used a New Mexico stock of *O. turicata*, none of which have ever been proved infective. In a recent experiment, early stage nymphs of this stock were not available, consequently specimens from another stock which had proved noninfective on two successive feedings were used. At the first test feeding following a blood meal on a white mouse infected with a spirochete foreign to *O. turicata*, spirochetes appeared promptly in the peripheral blood of the test mice. This cast suspicion on the "clean" stock being used. Thirty-five of the remaining ticks of this stock were, therefore, tested in seven groups of five each. All groups were shown to be carrying their own spirochetes. In some other instances the vicarious vector is not only infective but definitely infected.

As an illustration of the complicated problem of species determination, three geographic areas are chosen—Central Asia; Spain, North Africa, and Iran; and Panama, Colombia, and Venezuela. There are numerous species of *Ornithodoros* in the Central Asia area, but it is generally conceded by local workers that *O. tholozani* (= *O. papillipes*) is the common vector to man. It is found in parts of the U.S.S.R., Iran, Iraq, Palestine, Cyprus, India and has recently been reported from the western Egyptian Desert. Three names have been given to the borrelia transmitted normally by this species, viz., *Spirochaeta persica* Dschunkowsky, 1913, *S. usbekistanica* Pikoul, 1928, and *S. sogdianum* Nicolle & Anderson, 1928. None of these are accepted in *Bergey's Manual.*

Five specific names have been given to the borrelias transmitted by *O. erraticus*. These are *Spirochaeta crocidurae* Leger, 1917 from Senegal; *S. hispanica* de Buen, 1926 from Spain; *S. marocanum* Nicolle & Anderson, 1928 from Morocco; *S. microti* Rafyi, 1926 from Iran; and finally *S. merionesi* Blanc & Maurice, 1947 from Morocco. It will be noted that the origin of two names are geographic and that three are host names.

The third instance is in the Americas and concerns *Borrelia venezuelensis* (Brumpt) 1921 and *B. neotropicalis* (Bates & Saint-John) 1922. The former name was given to the spirochete transmitted by *Ornithodoros rudis* (=*O. venezuelensis*) in Venezuela and Colombia and the latter presumably to the organism normally transmitted by *O. talaje* in Panama. However, it was later found that the tick actually being used in Panama was *O. rudis*. *B. neotropicalis*, therefore, becomes a synonym of *B. venezuelensis*. From

an epidemiologic view it has been postulated by Clark (26) that *O. talaje* is a vector from animal to animal and that *O. rudis* is a vector from animal to man and from man to man. However, it has yet to be shown experimentally that these two vectors are able to transmit each other's spirochetes!

The two handicaps to the study of *Borrelia recurrentis* have been the weak susceptibilities of animals other than man and the ape to this species and the failure of the louse to feed readily on laboratory animals. In a series of interesting studies, Baltazard and his co-workers (27, 28, 29) in Iran seem to have solved these problems. They have found that newly-born animals, including the grey mouse, *Mus musculus*, the white mouse, the white rat, and especially the rabbit are very susceptible to infection with *B. recurrentis* as well as to tick-borne strains. The body louse feeds readily on the new-born rabbit. As the blood of the rabbit is toxic to the louse, the lice were fed on man subsequent to the infective feeding. Lots of *Ornithodoros erraticus*, *O. lahorensis*, *O. turicata*, and *O. parkeri* were allowed to engorge on newly-born rabbits heavily infected with *B. recurrentis*. Subsequent attempts to recover spirochetes from the ticks over a period of from seven to forty-four days resulted in failure. When lice were fed on rabbits infected with *B. microti* (Iranian) and *B. turicatae* and *B. hermsii* (Western U.S.A.) the organisms survived and retained their full virulence for baby rabbits for twelve, nine, and ten days, respectively, for the three species. Two strains of *B. recurrentis*, one from man and one from lice, were studied in several rodents. There was a rapid and definite transformation by rodent passage with an increase in pathogenicity for the respective rodents and a decrease for man. Following the second rodent passage, *B. recurrentis* was transmitted by the bite of *O. erraticus*, a native species. During this transformation the dark-field appearance of the organisms also changed from the monorefringent form, previously reported by by one of the authors as typical of *B. recurrentis*, to the double-contour appearance of the tick-borne species. It is the opinion of the authors that *B. recurrentis* had reverted to its original tick-borne type, after having acquired unstable characters by passage through man and the louse. The experiments were well controlled. The implications are obvious and may well explain the reservoir of the relapsing fever borrelias in the interepidemic periods. The tick has frequently been suggested as such a reservoir, but this is

the first time that experimental evidence has been presented to support this plausible theory.

Methods of infection.—The three possible means of infection are by the secretions of the salivary glands, the coxal fluid, and the material expelled from the anus. In some species this material may be considered as feces, while in *O. moubata* it is generally considered as the secretion from the malpighian tubules. The methods of transmission are not necessarily the same for all species of *Ornithodoros*. Larvae and first stage nymphs of *O. turicata* are capable of infecting a mouse in less than a minute following attachment, long before there is any coxal fluid. It must then be assumed that infection is by means of the secretions of the salivary glands. Kemp and his associates (30) were unable to demonstrate spirochetes in the coxal fluid of ticks of this species and the fluid was not infective for white rats. Under the rapid feeding condition, coxal fluid is seldom passed on the host. *O. hermsi* has no coxal fluid. Following feeding, a fine white crystalline substance appears at the openings to the glands. Most discussions have concerned *O. moubata* which secretes a copious amount of fluid while feeding and at times may pass "feces" on the host.

There are numerous reports on this subject. Hindle (31) believes that infection takes place by contamination of the bite wound by the "fecal" material which is washed over the puncture by the coxal fluid. Wolbach (32) concluded from histologic studies of infective ticks that spirochetes may be transmitted in any secretory or excretory product of the tick. Boné (33) reported large numbers of visible spirochetes in coxal fluid and was unable to infect white mice by the injection of fecal material. Wheeler (34) was unable to demonstrate spirochetes in the salivary glands of *O. hermsi* but the organisms were present in the gut contents for at least thirty-eight days following a blood meal. Ticks of this species do not pass feces while feeding. Feng & Chung (35) studied a French Congo strain of *Borrelia duttonii* in *O. moubata*. Fifty-two ticks were dissected over a period of forty-one days. Over this period it was found that spirochetes were regularly present. They were never found in the malpighian tubules or in "feces," but were constantly present in the salivary glands, the coxal glands "and their reservoirs," and in the nerve ganglion. We have repeatedly been able to infect white mice by injection of the coxal fluid of *O. moubata*. In one instance only fluid from the right gland was

infective and in another instance, in which the fluid was proved infective, the host mouse did not become infected. There may be other media of infection but there can be no doubt concerning the infectivity of the coxal fluid. Its infectivity is not constant but neither is infection obtained regularly following feeding of a known infective tick. This is in marked distinction to the regularity of infection in successive feedings of known infective *O. turicata*, *O. parkeri*, *O. erraticus*, *O. tholozani*, and *O. hermsi*.

Serology

Proteus XK agglutinins.—Relatively high agglutinin titers for *Proteus XK* have been reported in louse-borne relapsing fever. Elsdon-Dew, in his *Abyssinian Notes*, reported titers of 1:1,600 (36), and later he (37) reported that of six hundred and fifty cases in which borrelias were found, two hundred and eleven gave a positive *OXK* reaction in titers of 1:100 or higher. In one hundred and ten of these cases the Weil-Felix test was made every fourth day. A progressive rise in titer was shown and in one case the titer reached 1:6,400. There was a rapid fall in titer immediately after the last relapse and a month later the titer was less than 1:50. It was assumed that all cases were louse borne because of parallel occurrence with cases of epidemic typhus.

Robinson (38) found that the sera from 98 per cent of one hundred and sixty-eight patients in Addis Ababa agglutinated *Proteus XK* with titers up to 3,200. He concluded that a history of high pyrexia, with chills and *XK* agglutination, with no spirochetes in the blood was indicative of relapsing fever. In cases where there was a second rise in temperature, spirochetes were always found.

Zarafonetis, Ingraham & Berry (39) reported titers of 1:2,500 in Egypt, and Garnham, Davies, Heisch & Timms (40) found that the sera of thirty-eight of forty patients in Kenya agglutinated the Kingsbury strain in titers varying from 1:40 to 1:2,000 with a definite rise in titer in some cases.

Although *OXK* agglutination in high titer is considered diagnostic for tsutsugamushi disease, positive reactions have also been reported in experimental rat-bite fever (41) and in rabbits which have been injected with cultures of *Haemobartonella muris* (1).

It is common knowledge that *Proteus OXK* is notorious for its rapid reversion from the O- to the H-phase. In reference to ano-

malous reactions in various types of infection, attention has been called to the instability of suspensions of *Proteus X* which have been shipped long distances, especially in the tropics. The first to deteriorate is *OXK*, then *OX2*, while *OX19* remains relatively stabile (42). In certain laboratories in the United States, *OX2* has proved the most stabile member of the group.

Complement fixation. —This reaction, which has become of great value in the study of the rickettsias, has not been adequately developed for differentiating the relapsing fever borrelias, chiefly because of the lack of cultures for test antigens. Stein (43) has proposed a test for the diagnosis of relapsing fever. Saponin-treated blood from heavily infected experimental animals was used for the antigen. The author reports that these antigens are stabile and have broad affinities. *Borrelia duttonii* serum from white rats gave similar results with *B. obermeieri* and *B. hermsii*. However, in other instances there was a tenfold difference between homologous and heterologous sera. It would, therefore, seem that this test, with possible refinements, might well be used in the antigenic analysis of the numerous species of borrelias rather than as a diagnostic test.

In Egypt, Wahba (44) reported on the use of borrelia cultures and of alcoholic extracts of liver and spleen from patients dead of relapsing fever. Complement fixation was obtained in all of ninety-eight cases wherein the blood was taken during the first relapse, in 88 per cent of twenty-seven cases wherein the blood was taken after the fall in temperature following an original attack of six days, but in none of thirty-one cases where the blood was taken after the fall of temperature following an original attack of five days. The controls regularly gave negative results.

Wolstenholme & Gear (45) have recently reported successful results with a *Borrelia duttonii* suspension prepared from a culture of this organism in the developing chick embryo.

The Wassermann and Kahn reactions.—The Wassermann reaction has frequently been reported as positive in the relapsing fevers. In Garnham's series, the Kahn reaction was positive in only three of thirty-six sera and it was considered that these reactions were probably due to syphilis or yaws.

New species.—Spirochaeta latyschewi Soviev, 1941. *Borrelia latyschewi* (Soviev) (46). This is a spirochete of gerbilles, *Rhombombys opimus* and *Gerbillus eversmanni* in Fergana, Usbekistan.

Its specific vector is *Ornithorodoros tartakovskyi* Olenev, 1931. It differs from the human strain in this area in various biological characters but especially in its lack of virulence for guinea pigs.

Spirochaeta microti Rafyi, 1946. *Borrelia microti* (Rafyi). Rafyi (47) reported the recovery from mice, *Microtus sp.*, in Iran of a spirochete which was pathogenic for rats but not for guinea pigs or rabbits. The specific name was given to differentiate it from *S. persica*, the agent of sporadic human relapsing fever in Iran, normally transmitted by *O. tholozani*, and from *S. recurrentis* transmitted by the body louse.

More recently, this spirochete has been recovered from the blood of the gerbille, *Tatera indica;* the hamster, *Cricetulus migratorius isabellinus;* and from *Ochotona rufescens* and *Mus musculus bactrianus* (48). The normal tick vector of *B. microti* is *Ornithodoros erraticus.*

Spirochaeta harveyi Garnham, 1947. *Borrelia harveyii* (Garnham) (49). This organism was recovered from the blood of a grivet monkey, *Cercopithecus aethiops centralis* Neumann, captured in the forests of southern Mau, Kenya Colony. The morphology resembles that of other relapsing fever spirochetes of man. The average length in Romanowsky-stained specimens is seventeen microns. By dark ground illumination the ends appear pointed, the body is bounded by two parallel lines, and the coils number, on the average, eight. It is pathogenic for mice, rats, and monkeys, but not for rabbits or guinea pigs. It caused a mild infection in man. It gives no cross-immunity with *B. duttonii* or *B. recurrentis.* It is not transmitted by the mouse louse, *Polyplax serrata* Burmeister, by the human louse, *Pediculus humanus corporis* De Geer, or by the argasid tick, *Ornithodoros moubata* Murray.

It is interesting to note that *O. moubata* was given three test feedings, because the reviewer had failed to recover *B. duttonii* from *O. moubata* prior to the third test feeding following the infective feeding. According to the observations of Baltazard, the double contour as observed under the dark field would place this organism in the tick-borne group.

Spirochaeta merionesi Blanc & Maurice. *Borrelia merionesi* (Blanc & Maurice) (50). It was recovered from the argasid tick, *Ornithodoros erraticus*, and from the gerbille, *Meriones shawi*, at Goulimine, southern Morocco. It is pathogenic for the white rat but inapparent in the guinea pig, and without elevation of tem-

perature. The organism differs from *B. duttonii* in its slight pathogenicity for monkeys and the lack of virulence for man. It is similarly separated from the hispano-african type by its lack of pathogenicity for guinea pigs. It is considered that this organism is more closely related to the *B. duttonii* v. *crocidurae* of Dakar. The authors point out that it was first recovered in Morocco by Baltazard from the gerbille in 1937.

Spirochaeta novae caledoniae. There is an interesting isolated reference to relapsing fever in New Caledonia (51). Spirochetes were found in the blood of a female, forty years old, who had never been away from New Caledonia. Intermittent fevers are reported as not uncommon on both the east and west coasts and from nearby islands. Malaria is unknown. Lice were not found in relation to the patient. The name *Spirochaeta novae caledoniae* appears in the literature (52), but the origin of the term has not been ascertained.

New isolations.—Hoffman, Jackson & Rucker (53) have recently discovered a spirochetosis in turkeys in California but were unable to find a vector.

Burroughs (54) has demonstrated for the first time in the United States that *Argus persicus* is a vector of fowl spirochetosis in Texas.

Steinhaus & Hughes (55) have reported the isolation in western Montana of an unidentified spirochete from hen's eggs after inoculation with liver tissue from hens.

The Treponemas

Bergey's Manual accepts eight species in the genus *Treponema*, including the causative agents of syphilis, yaws, pinta, spirochetosis of rabbits, the nonpathogenic *Treponema microdentium* from the normal oral cavity, *T. genitalis* from the human genitalia, *T. calligyrum* from smegma, and *T. mucosum* which is reported as having pyogenic properties.

The etiologic agent of "Mal del Pinto" or "Carate" has been honored by five specific names in the short period of three years: *Treponema carateum* Brumpt, 1939; *T. herrejoni* Leon y Blanco, 1940; *T. pícter* Pardo-Castello, 1941; *T. pintae* Curbelo, 1941; and *T. americanum* Rossi, 1942.

As in the case of the relapsing fever borrelias and the leptospiras, there are adherents of the unity and plurality theories. In

a recent expression for the unitarian interpretation, Hudson (56) places syphilis, yaws, bejel, and pinta, as well as conditions described under numerous other terms, in the category of treponematoses, all caused by one organism, *Treponema pallidum*. He considers the several "diseases" as different manifestations under varying conditions of climate, environment, hygiene, etc. Unfortunately, little is known about the etiologic agent of bejel.

The immunologic responses as expressed by the several serological reactions are similar. In *Bergey's Manual* the Wassermann reaction is reported as negative in *T. cuniculi* infection in rabbits. However, McLeod & Turner (57) have found the Eagle flocculation test to be positive, and Turner (58) has reported the Wassermann reaction also as positive.

The various reactions of the several treponemas in experimental animals and man give some information concerning the biologic relations of these organisms. McLeod & Turner (59) have studied the reactions of rabbits to *Treponema pallidum*, *T. pertenue*, and *T. cuniculi*. In *pertenue* infections the rabbits developed local and generalized lesions, positive serologic reactions, and responded to treatment with neoarsphenamine. Individual lesions lacked the degree of induration observed in *pallidum* infections but, histologically, the lesions were quite similar to those of syphilis and yaws. It was concluded that the three organisms are closely related biologically, but that the host-parasite reactions indicate substantial differences. In a continuation of these studies, Turner, McLeod & Updyke (60) found that six months following the intratesticular inoculation of rabbits with *Treponema pallidum*, *T. pertenue*, or *T. cuniculi*, there was a considerable degree of cross-immunity to each of these organisms.

Padilha (61) found that it was possible to obtain initial lesions of pinta in yaws patients by injecting them with *T. carateum*. However, the initial lesions appeared later and progressed more slowly than reported for normal individuals. Based on the results of these experiments and similar experiments by other workers with yaws and syphilis, it is believed that there is a partial group immunity amongst these three treponematoses.

For the most part, animal reservoirs other than man and arthropod vectors play no role in the epidemiology of diseases caused by these organisms, but, in the case of yaws, Kumm (62) and Kumm & Turner (63) have presented evidence which strongly in-

dicates that in Jamaica the fly, *Hippelates pallipes*, acts as a mechanical vector. This fly was found to be the most common insect feeding on yaws lesions, and *T. pertenue* was easily demonstrated in the "vomit drops" and in the dissected insect, but could not be found later than forty-eight hours after the infective feeding. The organism did not invade the salivary glands, neither was there any evidence of cyclical development. Transmission from man to the rabbit by the fly was readily effected. Numerous references to former theories concerning fly transmission in other areas are cited.

The Leptospiras

Leptospirosis of the *L. icterohaemorrhagiae* type in man is usually associated with water or damp places which have been contaminated by the urine of rats. Since 1922, two hundred and twenty-two cases of the classical type and six of *canicola* infection have been reported from twenty-three States and the District of Columbia. The seventy-eight cases reported from the Detroit, Michigan, area indicate a possible failure in clinical and laboratory diagnosis in other areas (64). It has been suggested that the lack of correlation between the incidence of the infection in rats and the disease in man may be due to the leptospiricidal effect of the increasing acidity of the rats' urine. However, in some instances, leptospiras have retained their virulence for twenty-two days in water. A pH of between seven and eight is considered as optimum for survival.

Laboratory diagnosis and the determination of species are based on the isolation of the organism in laboratory animals and culture media and the numerous serological tests, such as the complement-fixation reaction, the adhesion phenomenon (Rieckenberg reaction), precipitin tests with specific soluble substances, Pfeiffer's phenomenon, the absorption of antibodies, the agglutination-lysis test, or the agglutination test alone. The absorption test distinguishes between specific and group agglutination.

Classification.—In 1938, Schüffner (65) recognized nineteen serologically distinct types. Randall (66) has recently mentioned sixteen species; van Riel (67) has listed thirty-one serological types from the literature, but concedes not more than four species; and *Bergey's Manual* accepts the free-living *L. biflexa* (Wolbach & Binger), 1914, and three pathogenic species: *Leptospira icterohaemorrhagiae* (Inada & Ido), 1915; *L. hebdomadis* (Ido, Hoki,

Ito & Wani), 1917; and *L. canicola*, attributed to Okell, Dalling & Pugh, 1925.

An example of some of the difficulties encountered in the determination of species based on geographical location, the clinical syndrome, pathogenicity for laboratory animals, animal reservoirs, and especially serological reactions, as illustrated in three areas, Brisbane, Pomona, and Ingham, along the east coast of Queensland, Australia, may be cited.

In Brisbane, only the classical symptoms of *Leptospira icterohaemorrhagiae* infection were observed and this species of leptospira was recovered from the imported sewer rat, *Rattus norvegicus*.

At Ingham, leptospirosis is endemic, chiefly among sugar cane workers, but at times approaches epidemic proportions. There were benign and serious cases with and without icterus. In one hundred listed cases [cf. Walch-Sorgdrager (76)], there were twenty-two cases of icterus and seven deaths. Several species of rats and the bandicoot were shown to be carriers, but the native rat, *Rattus culmorum*, was considered the principal one. Two distinct serological types of leptospira recovered in this area were designated as "Ballico" and "Zanoni" and later "tentatively designated *L. australis* A and *L. australis* B" [Lumley (68)].

In the third area, at Pomona, cases occurred each year among dairy workers. Illness was of a mild type with no fatalities. The organism recovered was quite distinct from the ones isolated in Brisbane or Ingham, both in its pathogenicity for guinea pigs and serological reactions. The reservoir of the disease was unknown (1942) but it was believed that this type of infection was common in pigs in south Queensland. This Pomona strain has also been elevated to specific status, *L. pomona*, by Derrick, 1942, as it differed serologically from all other known strains. Reports from Switzerland tend to confirm the suspicion of the pig as a reservoir of *L. pomona*. Gsell (69) studied fifty cases of human leptospirosis, all of which were strictly limited to areas where anti-*pomona* agglutinins were present in the sera of pigs.

Incidentally, "*L. suis*" infections in pigs and man in Argentina (70) and "water, leptospira type II" infection found in pigs and also in the herdsmen who bathed in the same water in which they washed their pigs (71) adds a definite porcine leptospirosis to the other types noted.

Perhaps the most comprehensive, stimulating, and contro-

versial recent report is that of van Riel (231 references) (67). This is first of all, as stated by the author, a contribution to the problem of unity or plurality of the leptospiras. The studies were made at Butembo and Kamituga in the region of Lake Kivu, Belgian Congo. There were three hundred and sixty-four cases for clinical study. Twenty-one strains were isolated, eighteen from patients, two from the rodent *Arvicanthis abyssinicus*, and one from water. By the use of specific immune sera for numerous strains and the agglutination-lysis test and saturation of antibodies, these twenty-one strains were divided into eight groups. One strain was serologically similar to *L. grippo-typhosa*, recognized by many writers as the specific cause of swamp fever in Europe, which, in turn, is serologically identical with the Moscow V strain, and incidentally, with Strain B of the Andaman Islands. Another group resembles *L. bataviae* from Indonesia and a third group, the Benjamin strain, also from Indonesia. Three groups were related to both *L. icterohaemorrhagiae* and *L. canicola*. There were two groups which represented new types. The groups related to both *L. icterohaemorrhagiae* and *L. canicola* are of special interest as most workers consider these as very distinct species. The water strain was pathogenic for guinea pigs.

In an analysis of the criteria used by the adherents of the plurality theory, it is pointed out that many of the conclusions are based on a very small number of observations. In the Congo series, there was no correlation between the strain of infecting agent and the clinical syndrome. Weil's disease, infectious jaundice with relapses, was reported as only one type and not the most common of this protean infection. Anicteric forms were frequent and especially frequent was the meningitic form. The author believes that the symptoms depend on the tropism of the infecting organism for the liver, kidneys, or the meninges. There are also inapparent infections with some organisms.

The criterion of pathogenicity for guinea pigs is considered discredited as strains which were highly pathogenic for this animal in the Belgian Congo were not infective for guinea pigs in Cape Town. Moreover, *L. grippo-typhosa*, which is generally conceded as only slightly pathogenic for guinea pigs, frequently produced jaundice with a high mortality.

Van Riel takes a midway position between the absolute unity belief of Zuelzer and the plurality theory of Schüffner. He recog-

nizes *L. biflexa*, *L. icterohaemorrhagiae*, and possibly *L. hebdomadis*, but not *L. canicola*. Furthermore, he believes that the water reservoir conception fits more clearly into the epidemiologic picture than does the animal reservoir conception.

The problem of the determination of species within this genus is obvious from the two limited illustrations given above for Australia, where the conditions are relatively simple, and for the Belgian Congo, where they are quite complex. The Indonesian and Andaman strains are further examples of the difficulties involved.

Esseveld (80) has recently summarized the information concerning the numerous isolations in Indonesia (Java, Sumatra, and Celebes), their identification by means of the agglutination-lysis reaction, and their relation to man and other animals. At first the name *Leptospira pyrogenes* was given to all of the strains, but as the work progressed several definite serological strains were encountered and some of these have been given specific status. Three seemingly important species are: *L. salinem*, which is responsible for most human cases on the east coast of Sumatra; *L. bataviae*, which constituted 87 per cent of the determined strains from *Rattus norvegicus* and is also a cause of human infection; and *L. javanica*, which was serologically determined in 99.4 per cent of 164 *Rattus r. brevicaudatus* examined. Strangely enough, no infections with *L. javanica* have been found in man.

L. bataviae is listed in the appendix in *Bergey's Manual* with the suggestion that it may be a synonym of *L. hebdomadis*. *L. salinem* and *L. javanica* are not listed.

L. hebdomadis and the strain "H.C." have been recovered from the dog in Sumatra, *L. bataviae* and *L. pomona* in Java, and the strain "Ballico" in Celebes. These were all from cultures from the kidneys. The recovery of *L. pomona* from the dog is of considerable interest as this species has been considered peculiar to pigs. *L. bataviae* was also recovered from the cat in Java.

It now seems that *L. mitis* Mino, 1938, as well as the new "species" *L. oryzeti* Babudieri, 1947 (72), both from northern Italy, are considered from a serological standpoint as synonyms of *L. bataviae*. The status of *L. mitis* Johnson, 1942, from Australia is, as far as the reviewer knows, undetermined.

Rodent reservoirs.—In addition to numerous species of rats, other rodents found to act as reservoirs are *Microtus montebelloi*

for *L. hebdomadis* in Japan; *M. arvalis arvalis* Pallas in the Netherlands, where 31 per cent of 479 mice examined were naturally infected with *L. grippotyphosa*, and *Arvicanthis abyssinicus* found infected in the Belgian Congo. *Apodemus speciosus* is reported as a reservoir of *L. autumnalis* A, considered by some as a synonym of *L. icterohaemorrhagiae*, and *Apodemus sylvaticus* has been found naturally infected in England and Italy. *L. bataviae* has been recovered from *Micromys minutus soricinus* in Italy.

Canine leptospirosis.—Canine leptospirosis has been recognized in Europe for more than thirty years and in the United States since 1923. *Bergey's Manual* attributes the specific name *Leptospira canicola* to Okell, Dalling & Pugh (73). These authors studied the disease in dogs in England but considered the organism as *L. icterohaemorrhagiae*. In Schüffner's paper, *Recent Work on Leptospiroses*, in 1934 (74), he stated, "On the strength of the divergent characters, Professor Klarenbeek and I propose to name this organism *Leptospira canicola*."

In a recent report on leptospirosis in Bohemia and Moravia, Jirovec & Stolcová-Sutorisová (75) point out that Professor Lukes of the Veterinary School of Brünn for the first time (1923) observed this organism in dogs and gave to it the name *L. melanogenes* and that, furthermore, in 1925, Klarenbeek announced the name as *L. icterohaemorrhagiae canis*. Walch-Sorgdrager (76) has discussed the position of *Spirochaeta melanogenes canis* Lukes. Stuart (77) has recently discussed this problem and does not believe that Lukes's claim for priority has been substantiated.

In the United States *L. canicola* was first recovered from dogs by Meyer and his co-workers in California in 1937 (78). Although *Bergey's Manual* states that *L. canicola* infection in dogs is characterized by uremia but not by jaundice, the California workers found icteric and hemorrhagic types. *L. icterohaemorrhagiae* was recovered from dogs in Washington, D. C., by Randall & Cooper in 1943 (79). Esseveld (80) has reported *L. hebdomadis* from dogs in Sumatra and the Australian "Ballico" type from Celebes.

The incidence of canine leptospirosis in the United States, as determined by serologic methods, is indicated by studies in several widely separated areas. Meyer, Stewart-Anderson & Eddie (81) tested the sera of forty-seven normal dogs in San Francisco, 34 per cent of which gave positive agglutination reactions for *Leptospira canicola* in serum dilution of from 1:100 to 1:300,000. In a

rural section the sera of twenty-eight dogs gave similar reactions in only 14.3 per cent of the cases. Green (82) examined the sera of three hundred and sixty-eight dogs in southern California and concluded that about one-fifth of the dogs showed evidence of infection. Incidentally, the sera of one hundred cats and four hundred and twenty-six human beings gave negative reactions with *L. canicola*, but the serum from one cat and three human beings reacted with *L. icterohaemorrhagiae*. In a survey in New York City, Tiffany & Martorana (83) reported no reaction to *L. canicola* in the sera of fifty-nine individuals whose work brought them into frequent contact with dogs. In the Lansing, Michigan, area, Newman (84) found that of five hundred samples of dog blood, 29 per cent gave definite reactions with *L. canicola* and 2 per cent with *L. icterohaemorrhagiae*. In Pennsylvania, Raven (85) made two similar studies, one in dogs from Philadelphia and one in dogs received from several medical schools. Of fifty dogs tested in Philadelphia, 28 per cent gave positive reactions, 22 per cent for *L. canicola* and 6 per cent for *L. icterohaemorrhagiae*. Of one hundred and five dogs collected from rural areas, 38.1 per cent gave positive reactions. It is generally considered that males become infected more often than females. In this series this held true for the urban dogs, 38.5 per cent for males and only 16.6 per cent for females, but the dogs from the rural section showed slight difference in sex incidence. It is remarked that the rural series contained more adult animals.

Despite the rather high incidence in dogs, the reported number of cases in man does not exceed thirty. This may be due to the difficulty of diagnosing *L. canicola* infection in man.

Much of the difficulty surrounding the isolation of *L. canicola* is based on the resistance of guinea pigs, rats, and mice to this organism. In 1942, Morton (86) used the Syrian hamster in the study of one strain of *L. canicola* and one of *L. icterohaemorrhagiae*. The latter species, when injected in 0.5 ml. amounts of culture grown on Schüffner's modification of Verwoort's medium, produced death in from five to eight days. When the same dosage of *L. canicola* was inoculated, heart blood was positive in 48, 72, and 96 hr., but this dose was not lethal for the hamster. Larsen (87) found the golden hamster (*Cricetus auratus*) susceptible to both species of leptospira and reported that identification of the or-

ganisms could be made on the basis of reaction in different animals.

L. canicola does not produce symptoms in young mice, causes loss of weight, afebrile reaction, and infrequently death in guinea pigs, but causes hemorrhages and death in hamsters. On the other hand, *L. icterohaemorrhagiae* causes jaundice, hemorrhage, and death in hamsters, young mice, and young guinea pigs.

Randall & Cooper (88) recovered *L. canicola* in two instances by the intraperitoneal injection of hamsters with the urine of dogs, and *L. icterohaemorrhagiae* from the dog for the first time in the United States by the injection of whole blood and urine intraperitoneally into young hamsters.

Bovine leptospirosis.—During the period 1933–1943, certain unsatisfactorily explained conditions, variously reported as hemoglobinuria, icterohemoglobinuria, idiopathic hemoglobinemia, enterotoxemic jaundice, some of which were without doubt leptospiral infections, occurred in cattle in Toronto, Canada (89), Australia (90), Kansas (91), and Oklahoma (92). Brite (91) considered the disease which he observed in Kansas to be entirely different from post-parturient hemoglobinuria and the hemoglobinuria produced by *Clostridium hemolyticumbovis*. Over a similar period, reports of definite spirochetal jaundice came from several investigators in the U.S.S.R. (93 to 98).

In 1944, Jungherr (99) described from Connecticut the first proved cases in the United States. In the following year, Marsh (100) reported the death of twenty-five purebred Hereford calves on one ranch in Montana of an acute disease characterized by hemoglobinuria. In liver and kidney sections stained by Levaditi's silver nitrate method, leptospiras were found. The fatal cases were in calves from about six to ten weeks old. Mortality was about 90 per cent. A year later, Mathews (101) reported a disease of cattle associated with leptospiras in three counties in Texas. Four forms of the disease were described and the pathology had much in common with that of leptospirosis in dogs. As the serum in three field cases and six experimental cases did not agglutinate either *Leptospira canicola* or *L. icterohaemorrhagiae*, it was considered that leptospira infection was not conclusively proved. In 1947 (102) an outbreak was diagnosed in a herd of twenty Hereford cows in central Illinois. (This report also refers to cases in New Jersey, 1944.)

Since 1944, there have been several reports from Palestine. Freund (103) reported on icterohemoglobinuria in cattle; Freund, Trainin & Malkin (104) on contagious jaundice; Bernkopf (105), Bernkopf, Olitzki & Sturzynski (106) demonstrated specific antibodies in the sera of nineteen patients, all of whom belonged to occupational groups having close contact with cattle. They also report the disease in sheep and goats. Btesh (107) has reported the infection of man by *Leptospira bovis* Bernkopf. The organs of fatal cases contained leptospiras, while the blood of patients recovering from the disease contained antibodies for *L. bovis* but not for *L. canicola* or *L. icterohaemorrhagiae*. *L. bovis* was not pathogenic for rats or guinea pigs. In this connection it should be remembered that Noguchi in 1922 (108) reported a small leptospira from the gastric mucosa of the ox but did not give it a specific name.

Therapy

The susceptibility of the pathogenic borrelias, treponemas, and leptospiras to certain drugs and antibiotics varies widely. The following discussion considers only some of the arsenic-containing compounds and penicillin.

Arsenicals.—It seems possible that the original concept of the arsenicals as specific in the treatment of the relapsing fevers was based on the therapeutic action of this drug in syphilis and the general use of the term "spirochetes" for the infecting organism in each instance. The standard textbook statements concerning the arsenicals in the case of the relapsing fevers are being seriously challenged.

In Palestine, Adler, Theodor & Schieber (109) found that in untreated cases of tick-borne relapsing fever the number of attacks varied from one to fourteen. The number of attacks did not seem to be influenced by the arsenicals, and, in some instances, the treated case ran a more severe course than the untreated. Francis (110) found that neoarsphenamine failed to show therapeutic value in the treatment of three patients infected with a Texas *Ornithodoros turicata* strain and considered that the opinion concerning this drug as a "specific" must be radically modified. A similar failure with mapharsen occurred recently (1947) at the Rocky Mountain Laboratory, Hamilton, Montana. The diagnosis was made at onset by finding the borrelias in the blood, and treatment was instituted promptly. There were four relapses at fourteen-,

seven-, eight-, and eleven-day intervals. In each instance borrelias were easily found and mapharsen given within a half-hour of the appearance of objective symptoms. The organism was identified as *Borrelia turicatae*, a human strain which had been carried in its specific tick-host, *Ornithodoros turicata*, since 1938.

In reports of sixty-eight cases of tick-borne relapsing fever in Tobruk by Cooper (111), in an unspecified number of cases, presumably of *Borrelia duttonii* infection, from Laurence Marques by Marques (112), and in forty-one cases of *B. duttonii* infection from the Transvaal by Merskey (113), it was generally concluded that the value of the arsenicals was far less than is usually accepted. In a study of four hundred cases of tick-borne relapsing fever in East Africa, Quinn & Perkins (114) found that in eighty patients treated with neoarsphenamine there was the same average number of two relapses as in forty-nine patients which received only symptomatic treatment.

In a very detailed account of *B. recurrentis* infections in Egypt, El-Ramly (115) reported on the use of neoarsphenamine, mapharsen, and several other drugs. In eighty-seven cases in which one dose of neoarsphenamine was used, the relapse rate was 9.2 per cent. There were no deaths. In one hundred cases, two doses of arsphenamine were given on two successive days. There were no relapses and no deaths. In a third group of fifty patients, three successive doses of 0.3, 0.6, and 0.6 gm. were given. The relapse rate was 12 per cent.

There were also three groups of fifty patients each in which mapharsen was used. Group I received a single dose of 0.06 gm., Group II, two doses of 0.06 gm. on successive days, and Group III, 0.03, 0.06, and 0.06 gm. respectively on three successive days. In Group I there was a relapse rate of 22 per cent while in Groups II and III there were no relapses.

In a series of 134 cases of *Borrelia recurrentis* infection in Chinese soldiers in Assam, Wolff (116) found that two doses of 0.04 gm. of mapharsen administered from three to five days apart proved very effective. The mortality rate of 11.9 per cent was considered as offset by the poor condition of the patients. However, in a second series of 251 cases of *B. recurrentis* infection in the same general area, Dillon & Fisher (117) concluded that "treatment can be as bad or worse than the disease." The standard treatment of 0.45 to 0.6 gm. of neoarsphenamine was at times followed

by profound shock and death. In these cases the optimum dose was found to be 0.3 gm. The mortality rate was 2.8 per cent.

The physical condition of patients—especially in the louse-borne type—the various dosages used, and the time of administration in relation to onset, crisis, and relapse makes an assessment difficult. It is obvious that, although the mortality rate in infections with *B. recurrentis* is higher than in many of the tick-borne strains, *B. recurrentis* is much more susceptible to the arsenicals than the tick-borne borrelias.

Penicillin.—In early experimental studies with this antibiotic, Heilman & Herrell (118) found that in *Borrelia novyii* infections in white mice there was a mortality of 75 per cent in untreated mice, with only a 4 per cent mortality in treated mice. Working with the same species of borrelia in white mice and white rats, Eagle (119) reported that 130,000 units or 100,000 units per kg. were required in the "cure" of 50 per cent of these animals, and approximately 400,000 units to cure 95 per cent. Meanwhile, Augustine, Weinman & McAllister (120) reported very favorable results in a small series of mice.

In further studies on *Borrelia novyii*, Cumberland & Turner (121) found that crystalline penicillin G was more active than F, X, or K. It has been suggested that purification may result in the loss of therapeutic properties, but Williamson & Lourie (122) reported that in relation to *Spirochaeta recurrentis* the partially purified preparation was not superior to the crystalline form.

In another case at the Rocky Mountain Laboratory, the course of which ran almost concurrently with the above cited case under "arsenicals," mapharsen was administered promptly at onset, but, as this drug was not well tolerated, penicillin therapy was instituted at the first relapse which occurred eight days later. During the following four days the patient received 800,000 units of penicillin. Despite this treatment a second relapse occurred ten days following the first. Borrelias were found in each instance. Penicillin therapy was again instituted and continued until a total of 2,840,000 units had been given. Thirty-seven days following the second proved relapse, while the patient was on leave, there was a clinical relapse. The infecting organism was determined as *Borrelia turicatae*, the same species and strain as in the mapharsen-treated case. The incubation period was eight days following the bite of a larval *Ornithodoros turicata*.

During the louse-borne epidemic in Cairo, Egypt, Ingraham & Lapenta (123) treated fifty-two patients with penicillin; fifty-three untreated patients acted as controls. A dosage of 25,000 units of penicillin was administered every three hours until 1,000,000 units had been given. The controls received only symptomatic therapy. There were no relapses in the treated patients, while relapses occurred in 87 per cent of the controls. As with the arsenicals, it seems that *Borrelia recurrentis* is much more susceptible to penicillin than some tick-borne species.

Heilman & Herrell (124) found that penicillin was of definite value in the treatment of guinea pigs infected with *Leptospira icterohaemorrhagiae*, and Larsen & Griffitts (125) obtained similar results in Swiss mice infected with this organism and in hamsters infected with *L. canicola*, when treatment was started early in the course of infection.

In the use of immune serum in the treatment of the disease in man, as is more or less generally practiced in some countries, a determination of "species" of *Leptospira* is of more than academic interest.

A resumé of the vast literature on treatment of the treponematoses is beyond the scope of this review. For some recent reports and reviews see Tompsett & Kauer (126), Reynolds & Moore (127), Reynolds (128), Varela & Avila (129), Eagle & Fleischman (130), and Eagle, Musselman & Fleischman (131).

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THE NEUROTROPIC VIRUSES

By Edwin W. Schultz

Department of Bacteriology and Experimental Pathology
School of Medicine, Stanford University, Stanford, California

Although viruses are characterized in part by their tendency to single out individual types of cells or tissues within the hosts they attack, it is frequently not possible to classify them satisfactorily on the basis of their individual tropisms. This applies also to the neurotropic viruses, many of which are not primarily neurotropic. Those which are encephalitic are as a rule not without some effect on the spinal cord; those which are myelitic are not entirely without effect on the brain. Some are clearly both (encephalomyelitic viruses). While a few appear to be strictly neurotropic, others involve nervous tissue only secondarily. Rabies and poliomyelitis viruses are two which have been regarded as more strictly neurotropic. However, the primary neurotropism of poliomyelitis virus may be doubted in the light of recent observations showing its frequent occurrence in the digestive tract in the absence of apparent neurological involvement. Moreover, the neurotropism exhibited by a given virus is not necessarily equally expressed in different hosts, with the result that some viruses may show themselves definitely neurotropic in one kind of host and essentially viscerotropic or pantropic in another. Some are transmitted by contact, others, by arthropods. While many can be clearly separated from each other on the basis of antigenic differences, some are antigenically interrelated. No classification has therefore been attempted in this review.

Among the more significant developments is a wider recognition of the role insects play in transmitting neurotropic virus infections, and a greater appreciation of the fact that the host range for some of these is broad and frequently clinically inapparent.

Rabies Virus

Much of the earlier literature on rabies virus has been covered in comparatively recent reviews (1, 2, 3).

Characteristics of the virus.—It is a comparatively large virus (100 to 150mμ). The particles of street and fixed virus appear to be

of uniform and constant size (4). It has been cultivated in tissue explants (5, 6) and on the chorioallantois of the developing chick (7, 8). Veeraraghavan (9) has recently reported the cultivation of the Paris strain of rabies virus on a lifeless medium and has concluded that the agent of rabies is not a virus in the accepted sense of the term. We have been unable, however, to repeat these observations with another strain of fixed virus (10).

Its partial purification by isoelectric precipitation has been reported (11). It is destroyed at 60°C. in five minutes, but withstands low temperatures well. In the frozen state, its virulence may be retained for twenty-five months. Dried slowly under ordinary conditions it soon becomes inactive, but like most viruses when dried rapidly and sealed, it remains active and virulent for long periods of time (12). In the dry form its resistance to heat is greatly increased (13). It is sensitive to the photodynamic action of methylene blue and to rays from a mercury vapor lamp.

It is quite sensitive to differences in pH and is destroyed more rapidly by increased acidity than by increased alkalinity. Its maximum stability is between pH 6.4 and pH 7. Glycerine plus refrigerator temperature have been widely used to preserve it, but it is preserved best by storage at subzero temperatures. In infected tissues, it is destroyed by a few days' exposure to ether, but strains differ in the degree of their susceptibility. It is moderately susceptible to phenol, formaldehyde, and most other disinfectants.

Employed as an immunizing agent, the virus stimulates the production of neutralizing and complement-fixing antibodies. This applies not only to living fixed virus but also to viruses which have been suitably inactivated with certain chemical agents (14, 15).

Its pathogenicity extends to a wide variety of animals, including birds. Infections, however, exist chiefly among carnivorous animals which ordinarily transmit them by biting while in the furious stage of the disease. Blood-sucking or vampire bats may transmit the disease (Trinidad rabies, mal de caderas of South America, and derriengue in Mexico). Infections in the vampire bat are frequently inapparent and animals which recover from clinically manifest infections may continue to carry the virus. This mode of transmission has now been recognized in regions of South America and Mexico (16, 17). The bat-transmitted disease usually takes the form of paralytic rabies.

As is true of many viruses, rabies virus shows variations. Those

observed include variations in serological and immunological properties, in ability to infect by peripheral routes, and in a loss of ability to spread centrifugally from the central nervous system (CNS) into peripheral nerves (18). The most widely-known variation is the classical one induced by brain-to-brain inoculation of rabbits (Pasteur's virus fixé). Such a fixed virus can also be induced by brain-to-brain passage of street virus in dogs. In either case, there is a progressive shortening of the incubation period to one which becomes stabilized at four to five days. Most strains of street virus can be converted in this way to fixed virus, but strains differ widely in the ease with which this may be accomplished. Some "mutate" immediately or are already "attenuated" (19), while others resist numerous brain-to-brain passages and appear to be nonmutable. Among the more mutable strains are those isolated from the paralytic Trinidad type of rabies. This transformation to fixed virus is generally associated with certain changes in biological properties. Fixed virus no longer induces the formation of Negri bodies or does so only feebly; it no longer spreads centrifugally from the CNS into the peripheral nerves, and it is relatively noninfectious for rabbits by the subcutaneous route. However, when repeatedly inoculated into rabbits via the sciatic nerve, a fixed virus may regain its capacity to induce the formation of Negri bodies and may to some degree recapture the ability to spread centrifugally into peripheral nerves—to induce what the French have termed "septineverite." An important difference between street and fixed virus therefore lies in a much greater ability on the part of the former to implant itself in peripheral nerves. Variations in the immunological powers of individual strains have been reported (20).

Pathogenesis and pathology.—The disease can be transmitted experimentally to a variety of animals and by various routes. It is held that in natural infections the virus spreads to the CNS via peripheral nerves. This view is supported by the fact that incubation periods tend to be longer following bites on the lower extremities than in areas closer to the CNS, and by a decided tendency for the virus to be restricted to the nervous system in infected individuals (21). After reaching the CNS the virus spreads centrifugally throughout much of the peripheral nervous system. Such a spread seems to account for its frequent presence in the salivary glands, and at times in other tissues.

While Negri bodies are demonstrable with considerable regularity in naturally induced rabies, failure to demonstrate their presence does not necessarily exclude infection with this virus. Some strains of street virus produce Negri bodies freely, others to a slight extent only or not at all.

Although there is wide agreement regarding the specificity of the Negri body, there is a difference of opinion as to its exact nature. It is held by some to be a protozoan parasite (22), by others a product of cellular degeneration (23), and by still others as a cell product of "physiological nature" (24). The occurrence of Negri bodies in nerve cells which appear to be essentially normal rather than degenerate seems in accord with the latter view (24). Although Negri bodies are found in natural infections in nerve cells only, they have been induced under experimental conditions in corneal cells as well. These cells, however, appear to be the only kind of epithelial cells in which Negri bodies can develop.

Clinical aspects.—In human beings, incubation periods ranging from two weeks to over a year have been reported. Usually these are three weeks or longer following bites on the face and five to ten weeks following bites on the leg. These long incubation periods are held to make prophylactic treatment in man possible.

The clinical course shows variations. In its classical form the disease is ushered in by a period of great excitability (furious rabies) and ends in extensive paralysis (dumb rabies). The disease may, however, assume a paralytic form from the beginning. In such cases the clinical course tends to parallel that seen in fixed virus infections in rabbits, with regard to short incubation period, paralysis from the outset, and frequent absence of Negri bodies, the latter a fact of considerable diagnostic importance. Rarely cases arise in which the clinical manifestations are spinal in type and suggest a landriform or transverse myelitis. Whatever the type, the outcome in most animals, including man, is invariably fatal. Recovery is so rare that one need not qualify this statement, though instances have been reported (25). Vampire bats are exceptions, in that these animals may recover from symptomatic infections or acquire latent infections which may persist for long periods of time. Whether latent infections may occur in other forms of wild life is unknown. Rabies virus has been recovered from the saliva of normal dogs, but such occurrences seem to be extremely rare (26).

Laboratory diagnosis.—An important advance in the identification of rabies infection in suspected animals was made by Webster & Dawson in 1935, when they suggested the substitution of white mice for rabbits in carrying out biological tests for its presence [see (3)]. Johnson (27) found that the mouse test yielded positive results in about 10 per cent of specimens which failed to show the presence of Negri bodies. It will be recalled that some strains do not produce inclusion bodies in infected animals, or produce them in small numbers only. The mouse test therefore closes this gap. Various strains of white mice from different sources have been found to serve equally well for this purpose (28). Their age, moreover, has not proved an important factor in the results obtained with street virus (29).

The problems relating to the demonstration of Negri bodies in suspected materials have been helpfully outlined by Stovall & Pessin (30).

Immunity relationships.—Natural susceptibility is widespread among warm-blooded animals. All mammals are highly susceptible; birds and fowl in general less so. Reptiles and fish are insusceptible. Individual dogs, and also rabbits, have been observed to resist experimental inoculation (31). The basis for these rare instances of individual resistance is unknown. Since recovery from rabies is virtually unknown, naturally acquired active immunity is equally unknown. An active immunity of some duration can be induced artifically, however. The vaccines which have been employed for the purpose fall into two groups: (*a*) living fixed virus (methods of Pasteur and of Hoegyes); (*b*) inactivated fixed virus, either chemically inactivated (phenol, ether, chloroform or formalin) or ultraviolet irradiated fixed virus (32, 33). Chemically inactivated virus suspensions have proved about as effective as living fixed virus preparations, judged by the average incidence of the infection in persons treated by individual methods (33, 34, 35). How much of this uniformity in the results is related to the intensive and prolonged course of the prophylactic treatments and how much to the merits of the vaccines is not clear. Neither is it clear as to how much the incidence of infection in man is actually reduced by prophylactic treatments. Hull (36) puts the estimated chance of an infection resulting from the bite of a rabid dog as low as 16 per cent. However, even this figure provides a strong argument for the effectiveness of prophylactic vaccination, since the average mortality

(of rabies) over a number of years in persons who received treatment has been between 0.3 per cent and 0.49 per cent (34). Webster (37) nearly a decade ago observed, however, that the vaccines then on the market differed widely in antigenic potency—apparently due largely to a progressive deterioration in antigenicity which may take place following inactivation. Suitably inactivated, and employed promptly enough, even a single dose of inactive virus may be trusted to protect dogs for periods of six months to a year (38). Inactivation by ultraviolet irradiation appears to cause less loss of antigenicity than chemical inactivation (33, 39).

Little is known regarding the mechanisms underlying acquired active immunity in rabies. It is known that virus neutralizing and complement-fixing antibodies appear in the blood of immunized animals (14) and that some passive protection against experimental infection in mice can be induced with antisera (40, 41, 42). It appears therefore that antibodies play a role, but whether these are supplemented by changes in the nervous tissue itself is still unknown.

Epidemiology.—Rabies is almost world-wide in its distribution but has been eradicated from some regions by rigorous measures. Its dissemination is by no means limited to dogs, cats, and other domestic animals. Indeed, its main reservoir appears to be in wild life, especially among carnivora, including foxes, coyotes, and wolves. In some regions the infection exists in blood-sucking vampire bats, which are known to be able to transmit the infection to horses and cattle, and at times to man. Dogs, however, are the most frequent source of infection in man.

Control measures.—Present views on measures for the control of rabies have been outlined in several recent publications (3, 35, 43). It is agreed that the measures should include regulations covering the transportation of dogs and other susceptible animals between states by common carriers, annual vaccination of dogs, and making the disease reportable in all states.

Poliomyelitis Virus

The literature bearing on this virus has become much too large to handle satisfactorily in a brief review. Although a comprehensive review appeared in 1941 (44), much work has been done since then. It will be impossible to cite directly many of the papers which have appeared during this period.

Characteristics of the virus.—In size it is among the smaller viruses. Ultrafiltration studies have indicated that it is a symmetrical particle of 8 to 12 mμ. Electron microscopic observations on considerably purified material have yielded contradictory results, however. Rod or thread-like particles have been reported (45); also symmetrical particles (46, 47). Attempts to cultivate monkey virulent strains in developing eggs have been unsuccessful (48).

It is among the more stable viruses and is able to survive for considerable periods of time in water, sewage, milk and dairy products. Certain strains have survived for years in 50 per cent glycerine at refrigerator temperatures. Melnick (49) observed no change in the original infective titers of two strains stored at subfreezing temperature for twelve months, either at − 20°C. or −70°C. The temperature required to inactivate the virus in aqueous suspensions, based on thirty-minute exposures, has ranged from 45 to 55°C., depending on experimental conditions. Various factors, such as its initial concentration and degree of purity, play a role in the rate of its inactivation by heat (50, 51). It is readily inactivated by ultraviolet light (52).

It possesses exceptional resistance to a variety of chemical agents (53) and is stable over a wide pH range (54). It withstands treatment with ether, basic lead acetate, and a variety of chemical agents in germicidal concentrations (53). It seems to possess a higher resistance to chlorine and hypochlorites than most bacteria (55, 56), but this may be open to reinterpretation (57, 58). Ozone appears more active than chlorine (55). It is susceptible to the photodynamic action of methylene blue.

Although chemical procedures for effecting its purification have been improved (59), much of this can be accomplished more successfully by ultracentrifugation. Certain strains have now been obtained in relatively high states of purity by this method, supplemented or not by chemical procedures (45, 60, 61).

Little is known regarding the constitution of the virus other than that it is a heavyweight protein, probably a nucleoprotein. In carrying out ultracentrifugal analyses of the purified MV strain, Loring & Schwerdt (60) observed that it consisted of relatively homogeneous particles with an $S_W^{20} = 62 \times 10^{-13}$; in the case of the purified Lansing strain studied later (61), $S_W^{20} = 83.5 \pm 7.35 \times 10^{-13}$, based on the use of the McBain & Lewis transparent ultracentrifuge. The particles exhibited a relative monodispersion,

showed no double refraction of flow, and behaved otherwise as symmetrical rather than asymmetrical particles. These observations are therefore at variance with those reported earlier by Gard (45), who observed asymmetric rod-like particles showing a tendency to aggregate reversibly in more concentrated suspensions. The sedimentation constants obtained by Gard also differ from those obtained by Loring & Schwerdt. The theoretical aspects of the above observations are discussed in a recent review of purified animal viruses by Beard (62).

Strains are known to differ in antigenic constitution, but knowledge of the extent of these differences is still fragmentary. The literature on this subject up to 1939 has been interestingly reviewed by Findlay (18) in connection with variation in viruses. Immunological differences among monkey passage strains have since been reported by Kessel, Moore & Pait (63). Jungeblut (64) and Dalldorf & Whitney (65) have observed serological relationships between certain mouse-adapted strains and strains of Theiler's virus.

Methods of carrying out neutralization and immunity tests have improved (66, 67, 68), but an agreement on procedures appears necessary if the actual differences in the antigenic constitution of strains are to be eventually resolved. There are now two reports on the application of the complement-fixation test, the first by Dalldorf & Whitney (65), the second by Loring, Raffel & Anderson (69). Should this test prove dependable, it will greatly facilitate future observations on strains of this virus.

Differences have also been observed in the pathogenicity of individual strains for laboratory animals. It is of historical interest that Landsteiner & Popper, in 1908, made their original experimental transmission of the disease to baboons, but monkeys, particularly Old World species, soon came into use and for many years constituted the sole experimental animals. Many attempts to transfer stock strains to common laboratory animals failed. However, in 1939, Armstrong succeeded in transferring the Lansing strain, then recently isolated in monkeys from a human case, to the Eastern cotton rat (*Sigmodon hispidus hispidus*) and from this animal to white mice. Similar attempts have proved fruitful with relatively few strains, however (70). This suggests that there are marked inherent differences among individual strains with regard to their capacity to infect animals other than primates and

that possibly only certain strains possess the capacity to adapt themselves to rodent tissue. Milzer & Byrd (71) recently reported that some product of autolyzed brain tissue facilitated the transfer of certain strains to Swiss mice, and favored the direct isolation in mice of several strains from infected human feces and spinal cord. In addition to cotton rats and mice, the Lansing strain has also proved infective for hamsters and for muskrats (72).

Transmission to monkeys was for a time believed possible only with certain species, chiefly Old World monkeys. The two species which came into wide use were *Macacus rhesus* (*M. mulatta*) and *Macacus cynamolgus*. However, a number of additional species have been found serviceable within the past few years, including certain South American and African monkeys (73 to 76). Chimpanzees have been employed in fair numbers, largely because of their high susceptibility to comparatively natural modes of exposure to the virus. Indeed, individual instances of naturally acquired poliomyelitic infections in chimpanzees housed in zoological gardens have been reported (77).

Pathogenesis and pathology.—The natural portal of entry of the virus in man has not been established. Up to a decade ago it was quite generally held to be via the olfactory nerve to the olfactory bulbs, and then to the brain. It had proved easier to infect rhesus monkeys by the intranasal than by the digestive route, and virus had on occasions been demonstrated in the nasopharyngeal washings of patients and contacts. However, examinations of the olfactory bulbs from fatal human cases had rarely been made (78). When this was finally done it was found that few presented evidence that the virus had entered by this generally assumed pathway. Even yet extensive histological examinations of the olfactory bulbs from fatal cases in man have not been carried out. An important reason for the almost exclusive attention given for a time to the olfactory pathway as the probable portal was the wide use of *Macacus mulatta*, a species quite resistant to infection by routes other than intracranial and intranasal. It was ultimately found that certain other species could be more easily infected by other routes. It was observed, for example, that the Javanese monkey (*M. cynamolgus*) could be infected by applications of recently isolated strains to the pharyngeal and tonsillar mucosa, or by feeding. Earlier claims had been made that this monkey could be infected by the digestive tract, but infections by accidental contami-

nation of the olfactory mucosa could not then be excluded. With the discovery that a previous intranasal irrigation with zinc sulfate solution served to prevent infection by the olfactory pathway (79), it became possible to exclude unintentional infections by this route. It is well established now that infections can be induced in some species of monkeys, and especially in chimpanzees, by the oral route. Moreover, Horstmann and her associates (80) have recently reported infections in two of seven infant rhesus monkeys given virus orally, showing that this species is not wholly refractory to infection by this route.

The species of monkey is not the only factor in establishing infection by the oral or digestive route. Much seems to depend also on the strain of virus employed, newly isolated strains having proved more dependable in this respect than old brain-to-brain passage laboratory strains. The most consistent results have been obtained in working with newly isolated strains in chimpanzees (81). That newly isolated strains are more highly infectious by peripheral routes than long term monkey passage strains is also suggested by the fact that the only two instances of laboratory infections in the long history of work with poliomyelitis virus have occurred recently in persons working with such strains (82).

The recognition that virus is commonly present in the stools of patients, and frequently in those of contacts, has further served to draw attention away from the olfactory mucosa as the likely portal and centered it on the oropharynx or the alimentary tract. The occurrence of virus in the stools of patients was in fact observed by Kling, Petterson & Wernstedt more than thirty years ago, but until a decade or so ago its presence was explained by most workers as virus which had been swallowed with the saliva. However, its regular presence in the stools, and often in high concentrations, can scarcely be accounted for in this way. Just where the virus does multiply and how it reaches the intestinal contents in such large amounts remains unknown. No histological or other evidence has been obtained to indicate its source. Attempts have, in fact, been made to determine whether perchance it may be able to multiply within the intestinal flora or fauna (83, 84). Wherever its multiplication takes place, it can apparently reach this site by routes other than the oral, since it has been found to make its appearance in the feces of chimpanzees and to set up "healthy" carrier states following inoculations by other than the oral route

(85). How the virus reaches the intestines under such conditions is unknown. Observations by Sabin & Ward (86) on the distribution of the virus in the tissues of fatal cases failed to show that the virus had spread centrifugally from the CNS to the intestines via the nerve supply of the latter.

Virus has been demonstrated in the oropharynx of patients in the early stages of the disease (87), and at times in the tonsils or the throats of symptomless children (88). A higher incidence of bulbar poliomyelitis seems to occur in children who have had recent tonsillectomies (89). However, observations suggesting that dental caries with pulp exposure may afford a portal of entry (90), have not been supported by recent studies (91).

The possibility that infection may occur at times via the skin has not been overlooked. The possibilities include transfer by insects or by ordinary contact, particularly when the skin is abraded. The latter possibility has received circumstantial support in one supposed case of accidental laboratory infection in man (82).

The sequence of events after the virus has established itself in the peripheral tissue is far from clear. There is a large body of evidence showing that the virus, once established in peripheral nerves, remains restricted to the nervous system and that its dissemination thereafter is effected along neuronal pathways. But it is not known where this initial invasion of the nervous system occurs. Recent studies have shown that in fatal cases the virus is regularly demonstrable in the walls of the alimentary tract as well as the CNS (86). Although the virus has been demonstrated at times in other tissues, its presence in other than these two sites is infrequent. It has recently been demonstrated to be present on occasions in the blood of patients (92), and in one instance it has been recovered from the blood of a convalescent (93). It has also been demonstrated in the blood of mice following intracranial or intraperitoneal inoculations with the Lansing strain (94), and in that of paralyzed monkeys (95) and of chimpanzees (96) infected with recently isolated strains. It seems probable, however, that these occasional appearances in the blood are the result of a "spill-over" from the primary foci and that this also accounts for the occasional presence of virus in tissues other than the CNS and gut.

Although it has occasionally proved possible to demonstrate lesions in the celiac ganglion of human cases and of chimpanzees following infection by the oral route, it has not yet been estab-

lished that invasion of the CNS ordinarily occurs via the nerves supplying the abdominal viscera. Bodian & Howe (97) have recently dealt with the significance of lesions in peripheral ganglia in chimpanzees and in human necropsy material in connection with the problem of interpreting the possible routes of infection and have stressed the dangers inherent in such interpretations. They point out: (*a*) that infiltrative lesions met with in sympathetic ganglia, especially in chimpanzees, are not necessarily related to a poliomyelitic virus infection; and (*b*) that the presence of lesions in sensory ganglia may mean a secondary centrifugal spread from the CNS. The problem is further complicated by the fact that spread of the virus by a given nervous pathway is not necessarily associated with obvious changes; moreover, the amount of virus initially present may drop to a level where it is no longer demonstrable. Bodian & Cumberland (98) observed in rhesus monkeys infected with the Lansing strain that while the maximum concentration of virus in the cord was reached on the first day of paralysis, a precipitous drop occurred within two or three days after the onset of paralysis. This may well be a disturbing factor in assays of virus made on human tissues intended to throw light on pathways of infection.

The possibility that natural strains may possess the ability to grow in nonnervous tissue cells has not gone unheeded (99). There are indeed good reasons for keeping it in mind. Among these is the tendency of virus to persist for a time in the stools of convalescents, its occurrence at times in the stools of healthy persons, and the ease with which stool carrier states can be created in chimpanzees with recently isolated strains (80, 100). It seems entirely possible that under natural conditions the virus is not a strict neurotrope and that its invasion of the nervous system is a purely accidental phenomenon rather than a necessary part of its host relationship. Its multiplication in nonnervous tissue need not necessarily be associated with obvious changes in the cells which support its growth. Myocarditis and other visceral lesions have been observed in fatal cases of human poliomyelitis (101), but whether the virus itself is in any way responsible for these lesions is unknown. Of some possible interest in this connection are the recent observations by Schmidt (102) bearing on the isolation of a virus apparently responsible for myocarditis in anthropoid apes and which on intracerebral inoculation into mice and hamsters

induced paralysis. It cannot yet be said, however, that these observations are in any way related to the myocarditis observed in human poliomyelitis, since it is known that many viruses which show little or no neurotropism under natural conditions induce CNS infections in laboratory animals when inoculated intracranially.

The pathogenesis and pathology of the paralytic form of the disease has been dealt with in a large number of publications. Among the more recent are those by Howe & Bodian (103), Peers (104), Ehrich & Foster (105), Elliott (106) and Bodian (107). All of the evidence points to the fact that once implanted in the nervous system the virus spreads within it along neuronal pathways. The lesions induced may vary widely in distribution and may be found in the brain and brain stem, as well as in the cord. In the cord the anterior horn cells are affected primarily. Here also the intensity of the lesions is subject to wide variations. The most characteristic lesions produced consists of a necrosis of the motor nerve cell bodies followed by a phagocytosis of the destroyed cells (neuronophagia). There are reasons to believe, however, that many of the cells attacked are not destroyed and gradually recover from the injury inflicted. The amount of impairment in muscle function induced is probably influenced very largely by the actual distribution of the individual neurones which have become involved in a given segment of the cord. Paralysis is not a necessary consequence of CNS invasion. Nonparalytic, as well as paralytic, poliomyelitis may be associated with CNS invasion, but in the former spread of the virus may be arrested at given points (108), or the virus if it does reach the more vulnerable motor nerve cells may not reach significantly damaging concentrations in the particular host (109). Strains which habitually show little tendency to paralyze monkeys when inoculated intracranially seem nevertheless to spread freely to the cord, but having reached it do little damage to motor cells.

Clinical aspects.—Manifestations of the disease range from extensive paralysis to a nonparalytic type of infection, the latter being far the more frequent. Diagnosis of the disease must be based almost altogether on clinical manifestations. Apart from certain nonspecific changes in the spinal fluid, there are no laboratory aids to which the clinician can turn. Although the virus is commonly demonstrable in the stools and sometimes also in the

oropharyngeal secretions, these examinations are time-consuming and costly. Improvements in the procedures for demonstrating virus in stools, sewage, or oral secretions have been recently reported (87, 110, 111, 112). There are no specific therapeutic measures at hand. Serum therapy was discontinued some years ago because of its doubtful value. Attempts to uncover a chemotherapeutic agent have proved unsuccessful (113, 114).

Immunity relationships.—Apparently all races of man are naturally susceptible to poliomyelitis. However, the incidence of the paralytic disease is not the same the world over. How much of this is due to differences in racial susceptibility and how much to climatic or other factors is unknown. Marked differences in individual susceptibility are apparent, however. It is generally held that a very small per cent of those who acquire the infection develop the paralytic form of the disease. Casey, Fishbein & Abrams (115) have estimated that perhaps 98 per cent of poliomyelitis infections occur as a mild disease leaving no residual paralysis. Why the few are more prone to the paralytic form of the disease is unknown. It may rest on some inborn differences in the protoplasmic constitution of nerve cells. Inheritable differences in susceptibility to given infectious agents among animals are well known. Attempts to relate susceptibility to paralytic poliomyelitis to the individual blood groups (116) have not served to throw any light on the role played by heredity.

One ordinarily includes as natural immunity those levels of resistance in which individuals or groups, although unable to resist infection entirely, get by with subclinical or abortive infections. Inasmuch as antibodies are generally elicited following subclinical as well as clinical infections, considerable use has been made of virus neutralization tests to determine the incidence of persons in different areas who possess such antibodies in their blood. Much of our present information on the distribution of the virus, including regions of the world where the paralytic disease is rare, is based upon such information. A high percentage of adults in various regions of the world have been found to possess antibodies for the virus. In earlier years these neutralization tests were carried out with monkeys as test animals, in more recent years, chiefly with the mouse-adapted Lansing strain.

Until about a decade ago it was assumed that the presence of neutralizing antibodies in the blood meant that an individual had

acquired resistance to the disease. It has become clear, however, that the mere possession of antibodies for a given strain does not necessarily signify that the individual is resistant to further infections, since patients with early poliomyelitis have been observed to have antibodies in their blood (117). Such situations may perchance rest wholly upon immunological differences among strains of the virus (118), but there is also the possibility that other factors play a role. Curious observations made during the past few years with the mouse-adapted Lansing strain as test virus suggest that much more work is required to clarify the significance of the presence of neutralizing antibodies in the blood for this particular strain, at any rate. It has been observed, for example, that although the incidence of persons showing antibodies for this strain is low in very young children and increases rapidly with age, it may be little changed during the course of an epidemic, even in persons known to have experienced an attack of the disease (119). Indeed, in place of showing a significant rise above the original level, such persons have been observed to sometimes show a decline in the antibody level for this virus (120). This also might conceivably be explained on the basis of antigenic differences among strains and the current prevalence of an unrelated strain. But to this puzzle has now been added the additional observation that antibodies for the Lansing strain may be demonstrated at times in the blood of dogs, cows, horses, chickens and wild birds (121), animals which so far have not been in any way identified with human poliomyelitis virus infections. It is possible, of course, that this strain is easily inactivated by nonspecific substances which may be present in some serums, especially when these are employed in low dilutions. Inactivation of poliomyelitis virus by occasional normal serums from poliomyelitis refractory animals such as the sheep, goat, horse and the Cebus monkey was in fact reported from time to time by earlier workers (122). Whether such results rest on a "maturation" phenomenon as expressed by Jungeblut some years ago or on exposure to some antigen shared by the virus cannot be stated. It is clear, at any rate, that an interpretation of the significance of neutralizing antibodies in the blood of normal persons, and of animals certainly, must await further studies.

Nutritional factors have long been considered as possibly related to resistance to infection. Studies on the possible roles played

by nutritional deficiencies in susceptibility to experimental poliomyelitic infections have not yet brought forth any results which might be considered of importance in dealing with the human disease (123). Attempts have also been made to determine the possible role played by certain endocrines (124, 125), by fatigue, chilling and mechanical trauma (126), and of seasonal factors (127) in susceptibility to experimental poliomyelitis. Observations on the "interference" phenomenon also have been extended to the experimental disease (128).

It is generally held that a frank attack of poliomyelitis leaves a lasting immunity to further attacks. While a well-defined immunity does result, it is not necessarily as solid as once believed, even in monkeys (129). Second attacks in man certainly occur (130). These may be due to immunologically new strains, rather than to a short-lived immunity to the original strain (118). Infection by a new route, if more than one is possible under natural conditions, might conceivably be another factor. It has been observed that immune animals may resist infection by a previously employed route but not by a new route (63). On the other hand, a well-defined immunity has been observed in monkeys and chimpanzees following subclinical infection with certain strains (81, 131), a fact of considerable practical significance in connection with the repeated use of symptomless animals. Such subclinical infections appear to be commonly associated with invasion of the CNS (132).

The mechanisms underlying acquired active immunity are not well understood. While neutralizing antibodies generally make their appearance in the blood soon after recovery, resistance to reinfection has been observed in monkeys before the appearance of antibodies in recognizable amounts. Moreover, antisera have not proved significantly protective when employed as passive immunizing agents against experimental infections (133, 134). It would appear therefore that tissue changes constitute an important factor in acquired active immunity to this disease. These seem to be localized, since subsequent inoculation by a new route may still cause infection (103).

There has been some renewal of interest in the possibility that active immunization against the disease may still be practicable. Results during the middle thirties had turned attention away from such endeavors. An important disturbing factor had been the heavy loss of antigenic potency on inactivation of the virus

by chemical agents, including formaldehyde. There have been reports lately, however, which appear to reopen the possibility of active immunization. Kramer & Geer (135), working with the Lansing strain in mice, found it possible to produce a significant degree of protection in these animals with mouse and cotton rat passage virus inactivated with formaldehyde. Loring and his associates (136), working with purified Lansing virus inactivated with formaldehyde, have reported a high degree of protection in cotton rats. Morgan, Howe & Bodian (137), however, found that while it was possible to induce a considerable degree of resistance in monkeys against intracerebral inoculation by intensive immunization with active Lansing virus, only inconclusive results followed the use of formalin-inactivated Lansing virus. Earlier, Milzer, Oppenheimer & Levinson (52) had observed that immunization with the Lansing strain inactivated by irradiation with ultraviolet light yielded a significant degree of protection in mice. Related to this problem of active immunization are the significant observations by Morgan (138) bearing on the distribution of antibodies in monkeys convalescent from poliomyelitic infections and in monkeys artificially immunized. She observed in monkeys which had passed through a paralytic infection an unusual mobilization of antibody in the CNS, while in monkeys which had been vaccinated intramuscularly with active virus and in which the blood showed a good antibody level, none was demonstrable in the CNS or spinal fluid. These results are helpfully discussed in her report.

Epidemiology.—We shall deal here only with observations bearing on the possible modes of spread under natural conditions. In dealing with the possible portals of entry, reference was made to the fact that while the virus had been demonstrated from time to time in the naso- and oropharyngeal secretions of patients, known contacts, and of normal persons, it had been demonstrated with greatest frequency in the stools of the three categories of persons mentioned. The two known potential sources of virus then are oral and fecal. The unsolved question is the mode of its transmission. Certain observations have suggested transmission by direct contact (139, 140), others some means of spread "other than contact" (141). Virus distribution studies seem to show that an outstanding feature of attacks on individual family groups is the occurrence of simultaneous infection from a common source, rather than a succession of infections such as might be expected from person to person contact (142, 143). Surveys have demon-

strated the occurrence of the virus, not only in the stools of patients, convalescents, and contacts (144, 145), but also in sewage (146, 147, 148) and in flies (149, 150). Horstmann and her associates (151) made observations on the length of time patients continue to excrete virus in the stools after recovery from the acute disease. It was found that while one of sixty-one patients excreted virus as late as the twelfth week, none of the group became persistent carriers. It was noted, however, that 61 per cent of the group excreted virus during the first two weeks after the onset of the disease. Similar observations have been reported by Brown and his associates (143). Melnick & Penner (152) have observed that blow-flies fed strains present in human stools showed virus in their excreta three weeks after the feeding, whereas mouse-adapted strains of poliomyelitis virus behaved like biologically inert material. The possibility that fly-contaminated food may constitute a mode of transmission is supported by the observations of Ward and his associates (153), who succeeded in infecting chimpanzees with food which had been exposed to flies at the homes of poliomyelitic patients within an epidemic area. An outbreak among naval cadets of polioencephalitis believed to have been food borne was reported in 1946 (154). However, while epidemiological data to date highly suggest that the disease may be food or water borne neither the portal of entry nor exact mode of transmission are as yet known.

Prevention.—It is futile to speak of definite measures of controlling a disease while its mode of transmission is still unknown. However, to the extent that we know the avenues of excretion of this virus, recognized measures can be taken against its possible dissemination from these sources. These constitute in essence all of the ordinary sanitary measures we recognize to be effective against enteric infections plus those one would employ in limiting respiratory infections. The latter is obviously more difficult to accomplish than the former, but should probably supplement ordinary sanitary measures as far as possible unless it does become definitely known that direct contact is not a significant mode of transmission.

Theiler's Encephalomyelitis of Mice

In 1934, Theiler (155) recognized the occasional occurrence of flaccid paralysis among stock albino mice and identified the causal

agent as a filterable virus. The ganglion cells of the anterior horns of the cord as well as isolated ganglion cells in the brain showed necrosis, but the virus did not prove paralytic for monkeys and was immunologically distinguishable from human poliomyelitis virus. Olitsky (156) later showed that the infectious agent is present in the intestinal contents of many normal mice. Although this disease is sometimes referred to loosely as "mouse poliomyelitis," there is no evidence that it is in any way related to the human disease. There are nevertheless some striking resemblances between this disease and human poliomyelitis (157).

The virus has a particle size of 9 to 13 mμ (158). It can be cultivated in developing hens' eggs (159), is a relatively stable virus and has been subjected to purification studies (45). Strains may differ considerably in virulence for mice and in infectivity by peripheral routes, the more virulent tending to be more markedly encephalitic (158). While there have been observations showing that strains of Theiler's virus are antigenically unrelated to strains of human poliomyelitis virus (157), this area should probably be more fully explored. Jungeblut has not only reported certain serological overlappings between his murine SK and MM viruses, and the GD VII strain of Theiler's virus (160), but also the curious fact that individual human sera neutralized the GD VII strain of Theiler's virus (161). Dalldorf & Whitney (162) have reported immunological relationships between MM, Lansing, and Theiler's virus.

A highly interesting feature of this virus infection is its common occurrence in the digestive tract in "normal" albino mice. The virus appears to be regularly absent in mice under twelve days of age, but after the thirtieth day a large per cent of them become carriers. It has been demonstrated not only in the feces, but in the intestinal wall, particularly of the duodenum, and at times in the mesenteric lymph nodes. It has rarely been demonstrated in the central nervous system in the absence of the paralytic disease. The latter moreover is of infrequent occurrence, since its incidence is not over one in three thousand to five thousand stock mice. How the invasion of the CNS occurs in these few instances is unknown. It is also not known where the intestinal virus multiplies. Its presence in the wall of the gut suggests that it may multiply here, possibly in the mucosa (157). Virus has been observed to be excreted in the feces up to fifty-three days after mice have been put

on sterile food and water (163), indicating that its immediate source is not exogenous. As mice increase in age, the virus output diminishes. Older mice, moreover, frequently show themselves immune to intracranial inoculation. Although such acquired immunity may result at times from a nonparalytic invasion of the CNS, evidence favors the view that the intestinal infection alone eventually leads to a humoral immunity sufficient to prevent infection by the intracranial route. Neutralizing antibodies for the virus can be readily demonstrated in the blood of older mice.

The clinical picture of the CNS infections is influenced to some extent by the virulence of the strain. While the strains ordinarily recovered from mouse feces induce a disease which is characteristically poliomyelitic in type, two strains have been isolated (FA and GD VII) which are somewhat more encephalitic and in general more virulent for mice than most strains (163). Histologically the paralytic disease resembles that observed in mice infected with the mouse adapated Lansing strains of human poliomyelitis virus (164).

Probably the fact of greatest practical significance connected with the recognition of this disease in mice is its bearing on the current use of this animal in the study of human poliomyelitis virus. Much confusion can result from accidentally picking up strains of Theiler's virus either in the course of passing bonafide mouse adapted strains or in attempts to adapt additional human strains to mice. While such possible sources of error may be guarded against by a strict adherence to certain criteria, they can nevertheless prove highly troublesome. This has recently been reemphasized by the observations of Melnick & Riordan (165). The possible occurrence of this virus infection in animals other than albino mice has not been fully surveyed. The virus has been recovered from wild gray mice trapped in animal quarters in which albino mice were known to be carriers (166), and from cotton rats and normal kangaroo rats presumably similarly exposed (167).

Teschen Disease of Swine

(Encephalomyelitis of Swine)

In 1929 a paralytic disease was recognized among swine in Teschen (Czechoslovakia). The disease has since been observed in Austria, Germany, Switzerland, and Yugoslavia. Its viral

etiology was established in 1933 by Klobouk (168). It has been briefly reviewed by Gard (45). Among the more recent papers describing it are those by Dobberstein (169) and Fortner (170).

The disease has been recognized to occur in an acute, subacute and chronic form. The acute disease, observed in sucklings, is frequently associated with pronounced encephalomyelitic symptoms. In the subacute and chronic forms flaccid paralysis of the limbs is the outstanding clinical manifestation, and in these latter two types, anterior horn cell degeneration is a prominent feature. Certain authors have emphasized the similarities between Teschen disease and human poliomyelitis (171). Dobberstein (169) has reported its transmission to apes. Hupbauer & Zarnic (172), on the other hand, were unable to show that human poliomyelitis virus is pathogenic for young pigs.

The disease can be readily transmitted to young pigs by intracranial or intransasl instillation, but has not been transmitted to other domestic or common laboratory animals. In infected animals the virus seems to be restricted to the CNS, but it may occasionally appear in the blood in low concentrations. It appears to be a moderately stable virus. Attempts to cultivate it on the chorioallantois of the developing egg have not been successful. It has been demonstrated in the feces during the prodromal stage of the disease, but not in nasal discharges. Animals which have recovered are not uniformly resistant to subsequent inoculations.

Avian Encephalomyelitis

A disease, sometimes referred to as "epidemic tremors of chickens," was first described by Jones in 1932 (173). It is characterized by tremors, especially of the head and neck, and by progressive ataxia. Chicks about three weeks of age are chiefly attacked. The CNS shows focal collections of glial cells and nerve cell degeneration, especially of the Purkinje cells in the cerebellum. The disease can be readily transmitted to young chicks by intracranial inoculation and is caused by a small virus (20 to 30 mμ) (174). Kligler & Olitsky (175) observed that while it multiplied to some extent in minced whole embryo tissue in Tyrode's solution, it failed to do so in developing eggs, in spite of the fact that it is a natural pathogen for young chicks. Chicks which have recovered from an attack of the experimental disease are immune to reinoculation and this immunity is associated with the appearance

of virus neutralizing antibodies in the blood (176). The literature on this disease has been reviewed by Jungherr & Minard (177).

Louping Ill, Russian Spring-Summer Encephalitis, and Australian X Disease

An encephalomyelitis of sheep in Scotland, characterized by cerebellar ataxia, was identified as a virus disease by Pool, Brownlee & Wilson (178) in 1930. It is transmitted by a tick, *Ixodes ricinus*. The infection is associated with a viremia but the CNS is by no means regularly invaded, many of the animals developing either an abortive or clinically inapparent infection. In those which do develop CNS involvement the characteristic lesion is an extensive necrosis of the Purkinje cells in the cerebellum, and this is largely responsible for the peculiar gait associated with the disease, the animals tending to spring up and down in moving forward.

The agent responsible for louping ill is a small virus, with a particle size of 15 to 20 mμ (179). It has been cultivated in developing eggs (180, 181) and is experimentally transmissible to mice, field voles and monkeys as well as to sheep. Infections may be induced by the olfactory pathway (182). Rabbits and guinea pigs are resistant. Mild influenza-like laboratory infections in man have been reported (183) and man is apparently definitely susceptible (184). In animals, recovery is followed by a well-defined increase in resistance to reinfection and by the presence of neutralizing antibodies in the blood. A significant degree of immunity can be induced by injections of formalin inactivated virus (185).

Casals & Webster (186) have recently reported a close antigenic relationship between Russian Spring-Summer Encephalitis, a tick-borne virus encephalitis of man in the timber country of Russia (187), and this tick-borne disease of sheep in Scotland. It is apparently relatively easy to establish a good immunity against both infections with formalin inactivated virus (185, 188).

In 1917–1918, a disease of man appeared in New South Wales referred to as Australian X disease. It was identified by Cleland & Campbell (189) as a neurotropic virus disease, experimentally transmissible to monkeys and to sheep. Inasmuch as the virus has not been available since 1926 and no undoubted cases of the disease have since then been reported, the exact nature of this virus disease has remained unsolved. The disease in question may have been either louping ill (190) or Japanese B encephalitis (*vide infra*).

Borna Disease

Borna disease is an infectious encephalomyelitis of horses and sometimes of cattle and sheep, which has been observed, and studied particularly on the European continent. The name "Borna" is derived from a locality in Saxony where a severe outbreak of the disease occurred during the years 1894–1896. It resembles but is clearly distinguishable from equine encephalomyelitis in this country. For comprehensive accounts of the disease the reader should read the excellent early review by Nicolau & Galloway (191) and the more recent one by Zwick (192).

Borna disease is caused by a relatively large virus, one measuring about 105 mμ. Attempts to grow it in tissue cultures have failed, and no effort appears to have been made to cultivate it in developing eggs. It is a fairly stable virus with more than average resistance to drying and other environmental conditions. Unlike strains of American equine encephalomyelitis virus, this virus appears to be antigenically homogenous. On experimental inoculation it is pathogenic for rabbits, guinea pigs, white rats, albino mice, and rhesus monkeys, the first being the animal of choice in experimental work. Results with chickens have proved irregular, while pigeons, dogs, and cats have proved refractory. The incubation period, both in the natural disease and in experimentally infected animals, is frequently long (fifteen to fifty days in rabbits). The initial symptomatology of the disease is related primarily to involvement of the cerebrum but this is often followed by a flaccid paralysis of the limbs.

The natural disease has been observed chiefly in horses, but what appears to be the same or a similar disease occurs also in sheep and cattle in the region referred to. The mortality in infected animals is high (above 90 per cent). Anatomically the disease is associated with a nonsuppurative inflammation involving especially the gray matter of the brain and to a lesser extent that of the cord. A distinctive feature is the very frequent occurrence of nuclear inclusions of Cowdry's type B (Joest-Degen bodies) in the large ganglion cells of Ammon's horn and to a lesser extent in nerve cells elsewhere.

The infection appears to be caused by a strictly neurotropic virus which not only spreads neurally to the CNS but also centrifugally from the CNS into the peripheral nerves. Its natural mode of spread among animals has not yet been determined. However,

the virus has rarely been demonstrable in the blood during infection, so that insect transmission probably does not occur. Since the virus has been observed to withstand ordinary drying for a considerable time, it is believed by some that a contaminated environment may possibly serve as a source of infection, whatever the portal of entry may be. The olfactory pathway has been considered a possible portal since virus has been demonstrated in the nasal and salivary secretions of infected animals. Although some measure of immunity against experimental infection has been induced with active virus, little or none results from inactivated virus suspensions. Neutralizing and complement fixing antibodies have been demonstrated in the serum of immunized animals but apparently no use has been made of virus neutralization tests to determine whether perchance this disease, under natural conditions, occurs in animals in a clinically inapparent form.

Unlike equine encephalomyelitis, no cases of Borna infection in man have been identified.

Equine Encephalomyelitis

For many years prior to 1930 a serious but unidentified disease in horses and mules was recognized in this country. In 1930, Meyer, Haring & Howitt (193) in California identified a virus as the causal agent. Shortly after this, Ten Broeck & Merrill (194) isolated a similar but distinguishable virus from animals on the east coast. Serological and other observations soon established that the two viruses are distinguishable, not only from each other but from the virus of Borna disease. The two American types have been designated Eastern and Western types respectively. A third, the Venezuelan type, has been identified more recently (195, 196).

The virus of equine encephalomyelitis by no means limits its attack to horses and mules but reaches out to a wide variety of mammals and birds. The infections induced in these animals frequently occur in a clinically inapparent form and appear to be wholly insect transmitted. Man also is susceptible and may suffer either a highly fatal encephalomyelitic infection or merely a mild or subclinical type of infection. Among the more recent reviews are those by Lépine (197) and Shahan & Giltner (198).

Characteristics of the virus.—On the basis of ultrafiltration studies carried out by Bauer, Olitsky & Cox in 1936, the particle sizes of both the Eastern and Western types were estimated to be

20 to 30 mμ. More recently, Sharp *et al.* (199), on the basis of electron microscopic observations and ultracentrifugal determinations on purified materials, have estimated the particle size of the Eastern type to be about 50.4 mμ and the Western type about 56.8 mμ. The particles were spherical with a rounded centrally-placed denser structure surrounded by a narrow zone of less dense material seemingly in the nature of a limiting structure. The material showed a high degree of monodispersion in the analytical centrifuge.

The virus is easily cultivated in tissue explants and in develope ing eggs (200, 201). Chemical studies of the purified virus have been reported by Taylor *et al.* (199, 202), who found it to be a liponucleoprotein complex of high molecular weight. It is stable between pH 7 and 10, but quickly inactivated outside of this range (203). In 50 per cent glycerol the virus may remain viable at refrigerator temperature for some months. It is inactivated by 0.2 per cent formalin, 1 per cent hydrogen peroxide, 0.2 per cent chloroform, and 3 per cent phenol. It has been reported to withstand 1 per cent phenol for several days and this concentration has been suggested as a preservative for brain tissue from animals in the field (204).

It has been mentioned that the Eastern, Western, and Venezuelan strains fall into distinct antigenic types (205). A comparison of strains of western equine encephalomyelitus have shown that considerable antigenic homogeneity exists among strains of this type (206). Various aspects relating to neutralization of the virus by antiserum have been dealt with by Merrill (207), Morgan (208), Labzoffsky (209), Whitman (210), and by Olitsky & Casals (211), and those relating to the application of the complement-fixation test by Casals (212) and others (213). The employment of tissue cultures for titration of the virus and for carrying out neutralization tests has been suggested by Huang (214). Koprowski & Lennette (215) have reported that neutralization tests carried out in developing eggs are about as sensitive as the intracerebral mouse test but less sensitive than the intraperitoneal mouse test (216).

A wide variety of animals are suceptible to experimental inoculation, including birds and fowls (217, 218). Guinea pigs and especially albino mice have been widely employed in experimental work. Rabbits are much less susceptible, in contrast with their susceptibility to Borna virus. Eastern strains have in general proved more

virulent than Western strains. The Venezuelan type, although highly infectious, is the least virulent. Natural infections also occur in a variety of animals, including birds. In many instances these infections occur in a mild or clinically inapparent form. In 1937 and 1938 a severe outbreak of the disease among horses in Massachusetts was associated with a considerable mortality among pigeons (219) and pheasants (220), all caused by the Eastern type. At the same time, severe cases of encephalitis in man, chiefly children, were traced to this virus (221). It has been clearly established since then that man frequently acquires the infection in a clinically inapparent form as evidenced by the presence of neutralizing antibodies (222, 223).

Pathogenesis and pathology.—There seems little reason to doubt that the disease is ordinarily transmitted by insects. In fowls expecially the virus makes its appearance in the blood, at least for a short period of time, from whence it may be easily acquired by blood-sucking insects. It is known, moreover, that infections with this virus can be readily induced by peripheral routes. The infection is probably as a rule a two-stage affair, an initial visceral phase being followed in some of the cases by involvement of the CNS (224). Many infections remain visceral only. When the CNS becomes involved, the brain and cord show little more in the gross than congestion and edema. Histologically, however, there are numerous scattered areas of neuronal necrosis, along with neuronophagia and perivascular infiltrations by mononuclear and polymorphonuclear cells. The pathology of the Eastern and Western types of infection in man is similar (225, 226).

Immunity relationships.—Perhaps the most striking aspect of this neurotropic virus infection is the large variety of mammals and birds susceptible to natural as well as experimental exposure. The recognition of this fact has depended largely on serological surveys, since the infections are frequently inapparent. In mice, susceptibility is influenced by the age of the animal. While both old and young mice are susceptible to inoculation with the Western equine encephalomyelitis virus by the intracranial route, only young mice are susceptible to the intraperitoneal inoculation (227). Older mice are also more easily immunized against experimental infection with equine encephalomyelitis, either Eastern or Western, than young mice (228). In the case of the Venezuelan type,

mice of all ages have proved susceptible to extraneural inoculation (229).

Recovery from infection, inapparent as well as apparent, is associated with the appearance of neutralizing antibodies in the blood. Active immunity can be readily induced by the use of formalin inactivated (230, 231, 232), or ultraviolet light inactivated virus suspensions (233). The effectiveness of this immunity appears to be related to the antibody level induced in the blood. That this plays an important role is suggested by the efficacy of passive immunity in preventing infection (234), and by the fact that specific immune serum has been shown to modify early experimental infections to a considerable degree (235, 236), a rare effect in experimentally induced virus infections.

Epidemiology.—The disease occurs almost altogether during the summer months. In 1933, Kelser (237) succeeded in transmitting the infection experimentally with laboratory infected *Aëdes* mosquitoes. By 1940, nine species of *Aëdes* had been reported as laboratory vectors (238); likewise *Dermacentor andersoni*, the Rocky Mountain spotted fever tick (239). Since 1940, the epidemiology of the Western type has been extensively investigated by Hammon and his associates. Their observations and others have recently been reviewed by Reeves (240), a member of the group. Widespread field observations involving surveys in which a large variety of arthropods have been examined for virus point definitely to *Culex tarsalis Coq* as the common vector in Western equine encephalomyelitis. The immediate source of these mosquito infections was obviously a large array of vertebrate animals, including domestic fowls and wild birds, which commonly acquire the infection in a clinically inapparent form. *Culex tarsalis* was found to feed principally on birds, as shown by precipitin tests on the blood taken up by the mosquitoes. Virus neutralization tests revealed that from 26 to 50 per cent of chickens in epizootic areas possessed neutralizing antibodies. Similar observations had also been made by Howitt & Van Herick (241), in the San Joaquin Valley of California. Hammon & Reeves (242) observed that a viremia could be induced regularly in chickens by the subcutaneous inoculation of the least amount of virus which would produce encephalitis in mice when inoculated by the intracranial route. In most instances this viremia was demonstrable between the twelfth and forty-

eighth hour, quite long enough for the virus to be taken up by a bloodsucking insect. The fowls showed no sign of illness. While *Culex tarsalis* appears to be the important vector of the Western type (243, 244), that of the Eastern type has not been determined. However, nine species of mosquito have been shown to be capable of transmitting the infection experimentally.

Shahan & Giltner (198) have recently reviewed the epizootiology of equine encephalomyelitis in the United States, while Gilyard (245) has reported recent observations on the Venezuelan type. Besides chickens, the duck, goose, and turkey have been found to acquire the infection naturally. Among wild birds natural infections have been observed in the pigeon, ring-necked pheasant, quail, Western robin, and the prairie chicken. Horses, mules, the cow, pig, sheep, goat, and dog may acquire the infection. Among wild mammals, infections have been found in the deer, jack rabbit, cotton tail rabbit, pocket gopher, and the brown rat. In most instances these infections are clinically inapparent.

Although effective vaccines have been developed for the immunization of horses, and of man, the problem of actually bringing the disease under full control is complicated greatly by the existence of the vast reservoir of infection from which arthropods may transmit the infection to man, horses, or other domestic animals. It is obvious therefore that adequate control measures must include control of the insect vector as well as active immunization against the disease. In the latter connection, it is important to recall that the strains causing these infections fall into distinct antigenic types, and since both Eastern and Western types have now been found in several States, both types may appear in areas where immunization measures have been taken against only one type (198, 246).

St. Louis Encephalitis Virus

During the late summer of 1933, an unusual outbreak of encephalitis occurred in St. Louis (247). This was caused by a virus which could be transmitted to occasional rhesus monkeys but not to rabbits (248), and to which albino mice later proved much more susceptible (249). Although no outbreaks of the dimensions of the St. Louis outbreak have occurred since, infections with this virus have been identified in different parts of the United States and other regions of the western hemisphere. A related virus disease,

Japanese B encephalitis, is known to have existed in Japan for many years (*vide infra*).

Characteristics of the virus.—It has a particle size of 20 to 30 mμ (250) and can be easily cultivated in tissue explants or in developing eggs (251, 252). It is a moderately stable virus and withstands storage for a time in buffered glycerine at refrigerator temperature or in the frozen state. Suspended in physiological saline at room temperature it soon becomes inactive. This inactivating action of saline is retarded by the addition of normal serum (253). It is most stable at pH 8.8 (254). It is readily inactivated by ultraviolet light (33); also by formaldehyde, but less easily by phenol. It seems to withstand Zephiran, Merthiolate, and sodium sulfathiazole in bacteriostatic concentrations (255). Antigenically it is related to but clearly distinguishable from the virus of Japanese B encephalitis and West Nile virus (256). Mice are much more uniformly susceptible to intracranial inoculation than rhesus monkeys. Not only albino but wild gray house mice are susceptible (257). Infections in both albino and wild mice have been induced by feeding the carcasses of dead or moribund infected mice, with subclinical infections in some of the animals so fed (258). Although rats and guinea pigs remain asymptomatic following intracranial inoculation, the virus has been observed to persist in the brains of these animals for eight or nine days (259). Similar observations have been made on baby chicks (260). Hamsters also have proved susceptible to experimental inoculation (261) and a number of domestic and wild animals are known to acquire the infection naturally, as a rule, in a clinically inapparent form.

Pathogenesis and pathology.—This is another arthropod transmitted encephalitic virus (*vide infra*). Although viremia in man has been reported only once (262), a well-defined viremia occurs in certain lower animals (263). Observations on its spread following inoculation by various peripheral routes in mice of different ages have been reported by O'Leary, Smith & Reames (264) and by Peck & Sabin (265). Although mice of any age can be readily infected by the intracerebral route, only young mice can be regularly infected by peripheral routes. The virus eventually becomes widely disseminated in the tissues of the infected animal—this in large part because of a centrifugal spread from the CNS during the late stage of the infection (265). The lesions produced in the natural disease in man are fully described in early reports (247,

266, 267). The CNS involvement is of the nonsuppurative type characterized by intense vascular congestion, cellular infiltration and degenerative changes in nerve cells. The lesions induced in experimental animals are similar (268).

Clinical aspects and laboratory diagnosis.—The clinical features of the disease are fully described in early reports (247, 269). Attempts to find a chemotherapeutic agent have failed (270).

As is true of most virus encephalitides, identification of the causal agent is impossible without laboratory assistance. Even then a direct demonstration of the virus during life is almost always impossible. The virus, with one reported exception (262), has never been demonstrated in the blood or spinal fluid of patients. The laboratory diagnosis must therefore rest on the appearance of antibodies in the blood of the patient. These may be identified by virus neutralization or complement fixation tests. To be significant, a serum sample procured early in the disease must be compared with later samples. The technical aspects of carrying out the neutralization test have been dealt with by Olitsky & Casals (211), and those relating to the application of the complement fixation test by Casals (212) and others (271). The use of the chick embryo protection test for demonstrating antibodies in the blood of man has been suggested by Blattner & Cooke (272).

Immunity relationships.—In addition to man, certain domestic and wild animals, including fowls and birds, are susceptible to natural exposure. In almost all instances, these infections are clinically inapparent; this is frequently true also in man.

Naturally acquired active immunity appears to be regularly associated with the presence of neutralizing antibodies in the blood. Active immunity against experimental infections can be induced with suitably inactivated virus (273), the immune state induced being associated with the presence of virus neutralizing antibodies in the blood. A passive protection in mice against intranasal inoculation may be induced (274). Young mice born of mothers actively immunized against the virus have been found to resist peritoneal inoculation (275). Passively immune mice may nevertheless acquire the infection in a clinically inapparent form. Slavin (276) found the virus in the CNS of mice five months after intranasal inoculation. The animals had been inoculated soon after they were born from hyperimmunized mothers. An active immunity against intracerebral infection can be induced by peripheral inoculation of active virus in older mice (273) and there are experi-

mental observations which suggest that immunization against the natural infection may be possible with inactivated virus suspensions (277).

Epidemiology.—The epidemiology of St. Louis encephalitis parallels to a considerable extent that of equine encephalomyelitis. It is an insect transmitted disease which frequently occurs in domestic and wild animals in a clinically inapparent form. Birds and especially chickens may constitute an important source of the virus in its transmission by insects. Several species of mosquitoes are known to be able to acquire and transmit the virus under experimental conditions (278, 279). It has, moreover, been isolated from certain culicine mosquitoes (*C. pipiens* and *C. tarsalis*) caught in nature (280). It has also been isolated from chicken mites (*Dermanyssus gallinae*) collected in nature (281). Infected mites have been found able to transmit the infection congenitally to their offspring, thus making it possible for mites to serve as a true reservoir of this virus (279). A possible epidemiological sequence which has been postulated is a congenital transmission of the virus in mites and from mites to chickens, and then from chickens to other vertebrates by mosquitoes (279). The virus has also been isolated recently from wild bird mites (*Liponyssus sylvirum*) in California (282). It may exist in arthropod hosts during nonepidemic periods. During an interepidemic study in two western areas, Hammon & Reeves (283) isolated three strains of St. Louis virus from *Culex tarsalis* and one from *Aëdes dorsalis*, the latter constituting the first isolation of St. Louis virus from *Aëdes* mosquitoes.

Japanese B Encephalitis

Outbreaks of "summer encephalitis" have occurred at intervals in Japan for more than three-quarters of a century. The qualification Japanese "B" encephalitis was introduced many years ago to distinguish this summer disease from encephalitis lethargica, a disease of unknown etiology, now of comparatively little importance in Japan or elsewhere. Particularly severe outbreaks of Japanese B encephalitis occurred during the summers of 1924 and 1935. The virus responsible was isolated during the 1935 outbreak (284, 285).

Characteristics of the virus.—It resembles the St. Louis virus in some respects but is clearly distinguishable from it. It measures about 20 to 30 mμ (286) and can be cultivated in explants of chick embryo tissue (287) or in developing eggs (288, 289, 290). It is antigenically clearly distinguishable from St. Louis virus but

in a measure related to it (256, 291). The Japanese virus, moreover, is encephalitic for sheep (291) and is in general of higher virulence for man and animals. Swine may develop characteristic encephalitis following intravenous inoculation (292), and hamsters are infected by ingesting the virus (293). Clinically inapparent infections, associated with viremia, have been induced in horses, goats, ducks, and chickens (292, 294). Chickens are easily infected by the subcutaneous route and may show a viremia for one to seven days (294).

Attempts to purify the virus have thus far not proved successful. Duffy & Stanley (295) were unable to purify it by ultracentrifugation because of the presence in mouse brain virus suspensions of tissue components having similar sedimentation constants. Its optimum pH stability range was found to be near pH 8.5. Even at pH 7 it was inactivated fairly rapidly. In titrating the virus, lower titers were obtained when Ringer's solution or phosphate buffer at pH 7, or saline phosphate buffer at pH 8.2, was employed than when 10 per cent rabbit serum in saline or in phosphate buffer was employed. Skim milk, adjusted to pH 8.4, was found a highly satisfactory diluent.

Pathological and clinical aspects.—The pathologic changes observed in fatal infections in general resemble those observed in other virus encephalitidides and have recently been dealt with by Zimmerman (296). A marked destruction of Purkinje cells with certain associated changes in the cerebellar cortex is often a prominent feature. This feature, together with its pathogenicity for sheep, suggests a relationship to louping ill, but the viruses responsible for the two diseases have been found to be antigenically distinct (291).

The encephalitis caused by the Japanese virus is more often fatal than that caused by St. Louis virus. The laboratory diagnosis is similar to that of St. Louis encephalitis and rests on a demonstration of the appearance of neutralizing or complement fixing antibodies in the patient's blood during the course of illness or convalescence (211, 212, 297, 298).

Immunity relationships.—Clinically apparent infections can be induced in mice, monkeys, sheep, swine, and hamsters. In certain other groups of animals infections are commonly inapparent. Both St. Louis and Japanese B encephalitis viruses have been observed to multiply freely in testicular tissue of guinea pigs

(284) or mice (299). In the latter, the Japanese virus displays a greater tendency to spread to the brain following intratesticular inoculation than does St. Louis virus (299).

A well-defined increase in resistance in mice to experimental inoculation can be induced with formaldehyde inactivated virus (300, 301), and such vaccines have also been observed to elicit the production of neutralizing antibodies in the blood of a varying per cent of persons (300 to 305). Although the responses have been by no means uniformly good, the results in general suggest that effective vaccination against the disease with inactivated virus suspension is possible.

Epidemiology.—The modes of transmission have not been fully determined. Mosquitoes are generally regarded as important vectors, but observations by Sabin, Ginder & Matumoto (306) suggest that these may not be the sole agents. The virus has been isolated from *Culex pipiens* and *Culex tritaeniorhynchus* collected in nature (307). Reeves & Hammon (308) found seven species of North American mosquitoes able to transmit the infection under experimental conditions. They suggest therefore that the virus could easily become established in North America, should it be introduced into areas where American encephalitidides are now endemic. The seven species able to transmit the infection experimentally included *Culex tarsalis*, *Culex pipiens*, *Aëdes dorsalis*, and *Culsieta inornata;* all of these are known to serve as vectors of the Western equine or St. Louis encephalitis viruses.

Sabin and his associates (306), in studies carried out in Japan, observed certain differences in the incidence of domestic animals showing neutralizing antibodies for Japanese encephalitis virus and those showing antibodies for St. Louis virus in this country. In contrast with the high incidence of neutralizing antibodies for St. Louis virus in chickens within endemic areas in this country, none of the fowls examined in two endemic areas in Japan showed neutralizing antibodies for the Japanese virus. There was, on the other hand, a high incidence of neutralizing antibodies in horses (84 per cent) and goats (70 per cent). Antibodies were also demonstrated in rabbits (33 per cent) and cattle (31 per cent). All of the animals tested had lived during five nonepidemic years in the two endemic areas studied (Okayama and Tokyo). Children seemed to have escaped infection, since only one of 62 tested showed neutralizing antibodies. Similar observations were made by Sabin (309) on

Okinawa. Here all of the fifteen Okinawan horses examined revealed neutralizing antibodies for the virus, as did three of ten goats and one cow, but none of twelve chickens examined. Among the natives, Sabin found that 90 per cent of those who were twenty years of age or older possessed neutralizing antibodies. The incidence was lower in the ten to nineteen year age group and none of sixteen persons in the one to nine age group showed antibodies. None of those showing neutralizing antibodies gave a history of encephalitis. The incidence of inapparent infection in both man and animals is therefore very high on this island, and since a viremia in animals may be demonstrated in clinically inapparent as well as apparent infections, its widespread dissemination may well be accounted for on the basis of insect transmission. Thomas & Peck (310) were able to demonstrate virus in the circulating blood of Okinawan horses three and six days after inoculation.

The existence of Japanese B virus has also been demonstrated in Shanghai and Tientsin (311). Recent cases of Japanese B encephalitis among American soldiers stationed in Korea seems to mark its first recognition in this region (312). Comparatively little is known, however, regarding its distribution in other regions of the world. "Autumn encephalitis" in the Maritime District of the Far East of the U.S.S.R. appears to be indistinguishable from Japanese B encephalitis (307). A curious observation has been the occasional presence of neutralizing antibodies in the blood of persons in regions in which the Japanese B encephalitis is not believed to occur, such as Canada and the United States (306), as well as Africa (313).

West Nile and Bwamba Fever Viruses

West Nile virus.—In 1940, Smithburn *et al.* (314) reported the isolation of a virus inducing encephalitis in experimental animals from the blood of an African woman who showed nothing more than a fever and who later developed neutralizing antibodies against the virus. Mice and rhesus monkeys inoculated intracerebrally with the virus developed encephalitis with lesions in the cerebellum similar to those seen in louping ill. Mice could be infected by the intraperitoneal as well as by the intracerebral and intranasal routes. Although virulent for rhesus monkeys by the intracerebral route, it caused only fever in certain African monkeys (*Cercopithicus ethiopocentralis*) and was not pathogenic for rabbits

and guinea pigs. Ultrafiltration studies showed it to have a particle size of about 21 to 31 mμ. Smithburn (315) later found the virus to be antigenically related to St. Louis and Japanese encephalitis viruses. It has since been established that although these three viruses are all related to one another, each is an immunological entity (186, 256). Smithburn & Jacobs (313) found many sera collected from residents in the Sudan, Uganda, Kenya, and the Congo active against the West Nile virus. Of even more interest are their observations showing that some sera from certain of these regions neutralized either the St. Louis or Japanese virus, or both, in a specific manner. Serum from one "blue monkey" caught in a forest in Kenya neutralized both West Nile and St. Louis viruses.

In addition to mice and rhesus monkeys, the virus is virulent for hamsters (316). It can be cultivated in developing eggs (317) and in tissue explants (318). A reduced virulence for mice and hamsters has been observed following cultivation by the latter method (318). Philip & Smadel (319) showed that *Aëdes albopictus* can transmit the infection from hamsters to hamsters by biting, and also that a few infected *Aëdes* given orally sufficies to transmit the infection to this animal.

Bwamba fever virus.—In 1941, Smithburn, Mahaffy & Paul (320) isolated another virus from the blood of natives in Africa ill with nonfatal infections characterized by fever, headache, backache, mild conjunctival congestion, and a skin rash. Inoculated intracranially into mice, the virus produced encephalitis with widespread degenerative changes in the pyramidal cells of the cortex. Monkeys developed a nonfatal febrile illness on intracranial inoculation, but guinea pigs and rabbits proved resistant. Ultrafiltration results showed that the virus has a particle size of 113 to 150 mμ. Convalescents showed neutralizing antibodies for the virus in their blood. All of the nine strains isolated appeared to be antigenically alike.

Neurotropic Viruses Isolated from Mosquitoes

Semliki Forest virus.—This virus was isolated in 1942 by Smithburn & Haddow (321) from *Aëdes abnormalis* mosquitoes caught in the Semliki Forest of Western Uganda. It proved pathogenic for mice by various routes, but for rhesus and red tail monkeys, rabbits, and guinea pigs only by the intracranial route. It differed immunologically from the viruses causing yellow fever, Bwamba

fever, St. Louis and Japanese encephalitis, Eastern and Western equine encephalomyelitis and from the West Nile virus (322). It was not known to cause illness in human beings, but a humoral immunity was demonstrated in a limited survey of persons from four widely separated areas. Antibodies for the virus were also demonstrated in individual animals representing six different species of primates. A high incidence of humoral immunity among *Cercopithecus nictitans*, together with its high susceptibility to inoculation, suggested that this species of monkeys may be frequently attacked by the virus under natural conditions (322).

Bunyamwera virus.—Recently also, Smithburn, Haddow & Mahaffy (323) have reported still another virus which appears to be new. This was isolated from *Aëdes* mosquitoes collected in the Semliki Forest in an area known as Bunyamwera. The virus proved encephalitic for white mice by various routes of inoculation. It differed immunologically from the viruses of yellow fever, Bwamba Fever, St. Louis and Japanese B encephalitis, the West Nile and Semliki Forest virus and several others. Neutralizing antibodies for it were found in the blood of a forest monkey, in a human being who had suffered a recent febrile illness with neurological signs, and in sera from a number of persons sampled at random.

Anopheles "A" and "B," and Wyeomyia viruses.—Roca-Garcia (324) has reported the isolation of three neurotropic viruses from forest mosquitoes in Eastern Colombia. All proved immunologically distinguishable from yellow fever virus. Only one, Anopheles A, was compared serologically with certain other known viruses. It differed from West Nile virus, and from Eastern and Western equine encephalomyelitis, St. Louis encephalitis and Japanese B viruses. All three of the new viruses isolated proved pathogenic for white mice on intracerebral and intranasal inoculation, but were not infectious for adult mice by the subcutaneous or intraperitoneal routes. The infections induced in mice by two of the viruses (Anopheles "A" and Wyeomyia) were associated with marked paralysis of the hind quarters, but infections induced by the third (Anopheles "B") were not especially paralytic. Baby mice proved susceptible to subcutaneous inoculation with the first two, but not with the last named. There were further differences which need not be mentioned here.

Ilhéus encephalitis virus.—During the course of a yellow fever

survey in Brazil in 1944, Laemmert & Hughes (325) recovered a neurotropic virus from the serum of a rhesus monkey which had been injected with emulsified mosquitoes caught in the vicinity of Ilhéus. The virus (serum from this monkey) proved encephalitic for mice on intracranial inoculation. It could also be readily transmitted from mice to mice by *Aëdes aegypti*. It proved serologically distinguishable from the Anopheles "A" virus of Roca-Garcia and differed from the latter's Anopheles "B" virus in being infectious for baby mice by the subcutaneous route. Antibodies for the virus were demonstrated in the blood of one of the investigators who had worked with it and also in three of sixteen other persons examined. None of these persons had experienced any illness suggesting a CNS infection. The virus was later characterized more fully by Koprowski & Hughes (326), who observed that it passed Seitz and Berkefeld W filters freely and could be easily propagated in tissue explants and in developing eggs. When inoculated into certain primates, marsupials, rodents, and birds, it failed to induce symptoms in most of the species tested. However, in certain of the species inoculated (e.g., marmosets) the virus persisted in the blood for periods as long as a week. The virus was found infectious for mice by a variety of routes. The infections induced were usually associated with a flaccid paralysis of the hind limbs and were not attended by convulsions or a period of excitement, such as characterizes Semliki Forest virus in mice.

"*California virus.*"—In 1945, Hammon, Reeves & Galindo (327) reported the isolation of a new virus from *Aëdes dorsalis* mosquitoes in the San Joaquin Valley of California, one showing neurotropism in mice. It was distinguishable from Eastern and Western equine, St. Louis, Japanese B and West Nile viruses. Neutralizing antibodies for the virus were found in the blood of man, horse, cow, rabbit, and squirrel in the region. The virus was later isolated from *Culex tarsalis* (283).

Herpetic Encephalitis, Pseudorabies and Ascending Myelitis or B Virus Infection

Herpes simplex virus.—This virus is a common, and ordinarily innocuous, inhabitant of the epithelium of the human mouth. Its relationships to such mild clinical conditions as herpes simplex, herpes febrillis and herpes genitalis are widely known. The reasons for including it here are three: (*a*) its well-defined neurotropism

when inoculated into certain experimental animals; (*b*) recent evidence showing that it may at times cause encephalitis in man; and (*c*) its close relationship to pseudorabies virus and B virus.

It is not the purpose here to review this virus in detail, but to refer briefly to certain observations which serve to characterize it and its relationships. More detailed accounts are available elsewhere (328, 329).

It is a comparatively large virus (100 to 150 mμ) which can be easily cultivated in tissue explants and in developing eggs. In the latter it produces characteristic focal lesions on the chorioallantois and frequently death of the embryos (330). It is a relatively unstable virus and fairly easily inactivated under artificial conditions. Strains of the virus appear to be antigenically homogeneous (329) and closely related to pseudorabies and B viruses (331). Although ordinarily a harmless parasite of man, its natural host, it is highly pathogenic for certain laboratory animals inoculated experimentally. Among the latter the rabbit is outstandingly susceptible and may be easily infected with most strains by a variety of routes. Applied to the scarified cornea it induces a keratitis, followed, in the case of most strains, by a spread to the CNS via peripheral nerves. Guinea pigs, mice, and rats can be infected by certain routes, but are in general less susceptible than rabbits. Rhesus monkeys seem to be in the main resistant, even to intracranial inoculations, but certain other species of monkeys have been infected by the intracranial route. A striking feature of the lesions induced by the virus is the presence of large acidophilic nuclear inclusions (Cowdry's Type A) in the involved cells, whether these be epithelial, corneal, or nerve cells. Similar inclusions are found in pseudorabies and B virus infections.

An outstanding feature of herpes simplex virus infection in man is its widespread occurrence as a latent infection (329, 332). In those who carry the virus, the infection is provoked into clinically recognizable form from time to time by appropriate stimuli, probably most often by factors which operate on the host side to upset the equilibrium in an otherwise well-balanced host-parasite relationship. Once acquired, the infection persists and, unlike most virus infections, clinical manifestations of infection may occur repeatedly in individuals once infected. In other words, an immunity to further clinical attacks is rarely, if ever, acquired. While virus neutralizing antibodies appear to be regularly and easily demonstrable in the blood of those who harbor the virus (329, 332),

these do not appear to play a significant role in preventing such clinical recurrences.

Infections with the virus apparently occur early in life while the oral epithelium is still young and tender. In 1938, Dodd, Johnston & Buddingh (333) brought out the important fact that most cases of aphthous stomatitis in young children are due to herpes simplex virus. Those who escape infection during earlier life, when susceptibility is high, apparently remain free of this infection during the remainder of their lives. Those who do acquire the infection appear to regularly develop a high antibody level for the virus, while those who fail to acquire this permanent infection show no antibody at all. It therefore becomes possible to identify those who are infected by virus neutralization tests, as observations by Burnet and his associates, and of others, seem to show (329, 332). It has moreover been found that a specific cutaneous reaction may be elicited for the virus in their blood but not in those who fail to show antibodies (334, 335).

Considerable uncertainty has existed over many years as to whether herpes simplex virus at times causes encephalitis in man. Although the virus had been isolated from a few cases of encephalitis lethargica, it had also been isolated from the brain and spinal fluid of patients with unrelated diseases, and also from the spinal fluid of apparently normal persons (328). The virus therefore came to be regarded more as a contaminant than a probable agent of encephalitis. Moreover, cases of encephalitis, in which nuclear inclusions of the herpetic type were observed, had not been established as definitely herpetic by actual isolation of the virus. Recently, however, four reports have served to establish the fact that the virus is at times responsible for encephalitis in man. In 1941, Smith, Lennette & Reames (336) isolated the virus from the brain of an infant with encephalitic lesions which were clearly associated with nuclear inclusions of the herpetic type. In 1943, Armstrong (337) isolated the virus from the spinal fluid of an adolescent male with aseptic meningitis who, following his recovery, was observed to have acquired neutralizing antibodies for the virus. More recently, Zarafonetis *et al.* (338) have also reported the isolation of the virus from a fatal case of encephalitis in a young adult in which the characteristic nucler inclusions in the affected brain cells again were demonstrable. Still more recently, Whitman, Wall & Warren (339) have reported two additional cases.

For a review of much of the literature relating to the neuro-

tropism exhibited by this virus in experimental animals the reader is referred to van Rooyen & Rhodes (328). Among the more recent papers on experimental herpes simplex virus infection in animals are those by Slavin & Berry (340), Berry & Slavin (341), Good (342), and Florman & Trader (343).

Pseudorabies virus.—In 1902, Aujeszky, in Budapest, inoculated rabbits with emulsions of material from a calf and a dog which had succumbed with rabies-like symptoms. The inoculated animals succumbed within two to three days after showing signs of severe pruritus followed by paralysis. The short incubation period and symptomatology clearly distinguished the disease from rabies. Infections with this virus have since been reported from a number of European countries and from both North and South America. The disease is also referred to as Aujeszky's disease, mad itch, and infectious bulbar paralysis. The earlier literature has been reviewed by Boecher (344) and by Bailly (345).

The virus seems to have the same particle size as that of herpes simplex virus (100 to 150 mμ). Nicolau & Motoc (346) have recently reported the recognition of virus elementary bodies within the characteristic nuclear inclusions produced in this disease. It can be cultivated in tissue explants and on the chorioallantois of developing eggs. On the latter it induces characteristic focal lesions with early death of the embryo (330), the virus invading the CNS of the embryo (347). It is easily destroyed by heat (60°C.), withstands drying fairly well, and may be preserved for many months in 50 per cent glycerine at refrigerator temperature. The virus is immunologically distinct from that of rabies, but related to herpes simplex and B viruses (331). Individual strains appear to be antigenically alike. It is infectious for a variety of animals under natural conditions and is easily transmitted experimentally to most laboratory animals. Except in swine, mortality is usually high. It is generally regarded as noninfectious for man, but at least one abortive infection in man has been reported (348).

The natural modes of transmission are not well known and appear to differ with the species of animal involved. Among rats and swine ingestion of infected rat carcasses appears to be a common mode of transmission, while in cattle skin contact appears to be the usual mode. Most animals can be readily infected experimentally by various routes. But the virus does not behave in quite the same way in different species of animals. In the rabbit, for ex-

ample, it is pantropic, while in the monkey it is strictly neurotropic. In either case, its spread to the CNS is via the peripheral nerves, as in the case of experimental herpes simplex virus infections, which it resembles closely (349).

The lesions induced differ somewhat with the species of animal. Inoculated into the lower extremity of a rabbit, the virus induces an intense local inflammation associated with tissue necrosis. There is an early involvement of the peripheral nerves, followed shortly by involvement of the corresponding spinal ganglia and segments of the spinal cord. The virus then travels cephalad and death ensues when it reaches the medulla. Visceral lesions also are frequently induced. Injected intravenously, the virus readily establishes itself in various organs. In natural infections in cattle, the involvements parallel those observed in experimentally infected monkeys in being primarily neurotropic. Nuclear inclusions of the Cowdry A type seen in herpes simplex infection have been observed in a variety of cells.

The clinical manifestations differ. In most animals the disease runs a stormy and rapidly fatal course; in swine, however, the infection is either mild or subclinical. In more susceptible animals, the onset is sudden and characterized by an intense pruritus ("mad itch"). Paralysis and death follow the onset within one or two days. Unlike rabies, the incubation period is short (two to three days).

Among domestic animals, swine, cattle, cats, and dogs are most subject to natural infection. Rats and mice and other wild animals may acquire the infection. Sheep and horses are more resistant. Among experimental animals rabbits are most susceptible, guinea pigs considerably less so, and birds and primates still less. Animals which have recovered from the infection are immune to further inoculations for at least 9 months. Acquired immunity is associated with neutralizing antibodies in the blood and these can be readily increased by further injections of active virus into recovered animals. Strains are antigenically homogenous and acquired immunity appears to extend to all strains of the virus. It has not proved possible to immunize against experimental infections with inactivated virus suspensions. Passive immunization also has not proved significantly protective.

Infections with this virus have been identified in various parts of Europe and in North and South America. In this country it

has been observed particularly in the Middle West (350, 351, 352), where it appears to be highly prevalent among swine as a clinically inapparent infection (350).

Ascending myelitis or B virus.—In 1934, Sabin & Wright (353), in New York, reported a remarkable instance of a fatal infection in man following the bite of an apparently normal *Macacus rhesus* monkey. Death followed an acute ascending myelitis. Focal lesions were also demonstrable in the patient's viscera (spleen, adrenals, and regional lymph nodes). Attempts to transmit the disease to *Macacus rhesus* monkeys, dogs, mice, and guinea pigs with glycerinated tissues from the case proved unsuccessful. However, on inoculating rabbits, a strongly neurotropic virus was demonstrated in tissues from the brain, cord, and spleen. Following intracutaneous inoculation in rabbits it induced a disease which strikingly resembled that seen in the patient. The nature of the lesions induced definitely distinguished it from the viruses of poliomyelitis, rabies, vaccinia, and certain others, except herpes simplex virus from which it was not then fully differentiated.

The virus was later studied more fully by Sabin (354) at the Lister Institute in London. His studies at this time showed that while it was immunologically distinguishable, it bore a relationship to herpes simplex virus and to pseudorabies virus. It could also be distinguished from the latter two viruses on the basis of differences in the evolution of and character of lesions induced in rabbits. The virus was pathogenic for guinea pigs but only irregularly fatal even after intracerebral inoculation. Although rabbit passage virus proved encephalitic for some mice, several attempts to pass it serially in mice failed. The virus proved encephalitic for rhesus monkeys by the intracerebral route but not by peripheral routes, in which respect it differed from rabbits. Acute local inflammatory reactions were induced, however, in monkeys at the sites of injection. The serum of one of thirteen "normal" monkeys was found to show a high level of neutralizing antibodies for the virus. The opinion expressed by the author that the failure experienced by Sabin & Wright in their attempt to transmit material from the patient to rhesus monkeys might have been due to an acquired immunity in the majority of monkeys employed in New York. Two additional monkeys were later found to possess antibodies for the virus. Burnet and his associates (331) in Australia

later found that individual cynomolgus, as well as rhesus monkeys, may show neutralizing antibodies.

It should be added here that Sabin found the virus readily filtrable through Berkefeld "V" and "N" and Chamberland L_3 candles. It can be readily cultivated in developing eggs, in which it induces lesions similar to those produced by herpes simplex virus (330). Its antigenic relationship with herpes simplex and pseudorabies viruses was referred to in dealing with these viruses (331). It has been postulated that the three viruses may have sprung from a common stock and have become specialized for their respective host species—herpes in man, B virus in monkeys, and pseudorabies in the pig (329, 331, 332). B virus infection appears to be endemic in monkeys, just as herpes is in man and pseudorabies is in swine, the infections in all three instances rarely being at a clinical level within the respective natural hosts. While in all three instances the viruses are comparatively innocuous to hosts in which they occur naturally, all three are highly fatal to other animals, all three are predominantly but not strictly neurotropic, all three produce similar nuclear inclusions, all three invade the CNS via peripheral nerves, all tend to produce death in experimentally inoculated animals at a time when little virus is present in the brain (331). Moreover, a primary myelitis may be induced in rabbits following intravenous injections of herpes simplex virus (355, 356).

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BACTERIA AS PLANT PATHOGENS

By Walter H. Burkholder
Department of Plant Pathology, Cornell University
Ithaca, New York

The recognition that bacteria cause diseases in plants was relatively slow in establishing itself. That fungi were the causal agents in many instances had been accepted by botanists in the middle of the nineteenth century, and the next step, that of demonstrating the phytopathogenic nature of certain bacteria would normally seem to follow. It was not, however, until 1900 that all skepticism and even open ridicule of the few investigators in the field vanished and the work of the eighties and nineties was evaluated without prejudice.

Although a few observations had been made earlier on bacteria associating with disease symptoms in plants, the publication of Burrill's work (1) in America on the fire blight of apple and pear in 1882 and that of Wakker (2) in Holland on the yellows of hyacinth the following year are usually considered the birth of the science of bacterial phytopathology. These two papers and those that follow by the same authors, and the continuation of Burrill's work by Arthur (3), are excellent for their time; but they had little influence on the thinking of contemporary workers, and approximately twelve years elapsed before other papers of their caliber began to appear in the scientific journals.

This slow progress of the science during these years may be attributed, without doubt, to the adverse or at most, luke warm attitude of some of the most renowned and influential botanists of their day. A picture of this period, including the work of the few investigators and the opinion of the eminent biologists concerning the work, has been given in an interesting review by Smith (4). Dr. E. F. Smith, who is now considered the father of bacterial phytopathology, had entered the field early in the nineties and by 1898 had proved the bacterial nature of five of the more troublesome plant diseases (5). A perusal of these papers at the present time leaves one very much convinced of their veracity and logic, but a certain group of biologists in Europe were still skeptical, and led by Fisher (6) they more than questioned Smith's results. An extremely

heated argument ensued between Fisher and Smith which lasted two years. The articles published in the *Zentralblatt für Bakteriologie* (7 to 10) are of interest in giving an insight into the thinking of the time and for Smith's presentation of the foundation for work in the subject. After this dispute, if anyone still doubted the phytopathogenic nature of various bacterial species it was not recorded in print. That Smith had emerged as victor was shown by the number of papers on bacterial plant diseases that began to appear in the botanical periodicals at the beginning of the present century. They came from American and European laboratories alike and are still appearing supplemented from workers throughout the world.

In the United States, investigations on the bacterial diseases of plants centered for some years in the Bureau of Plant Industry at Washington, D. C. Here, a group under Smith's direction produced some of our best work during the first quarter of the century. After Dr. Smith's death in 1927 the members of his office were allocated to other departments and now many of them have retired. No one institution has ever taken the place of this office, but scattered investigators in the universities and experiment stations of the United States and Canada for the last fifty years have contributed to the science, at times with extensive investigations, and at times filling in the gaps of previous work.

In Europe, Paine, Lacey, and others from the University of London have contributed an extensive series of articles entitled *Studies in Bacteriosis*. For the most part, however, investigations came from individuals at many institutions. In Asia a number of Japanese plant pathologists have published on the subject and there have been numerous papers from India, Australia, New Zealand, South Africa, and Hawaii. These workers have presented a comprehensive survey of the bacterial diseases of cultivated plants that is world wide. Little, however, is known concerning the bacterial diseases of wild plants.

Classification

In the sixth edition of *Bergey's Manual of Determinative Bacteriology* there are now listed approximately two-hundred species and varieties of bacteria causing plant diseases that have been described sufficiently well for one to identify with certainty. The extent and validity of these descriptions compare favorably with

those of other bacterial species entered in this *Manual.* Some fifty other species have been reduced to synonomy, and an equal number of binomials may be found in the appendices of various genera which are *nomen nudum;* or if a description does exist in the literature, the pathogenicity of the species may not have been adequately proved. To a medical man or to a veterinarian the number of these phytopathogenic bacteria probably seems large, but the number of species of plants under cultivation is vast in comparison with the domesticated animals. Comparatively the number of these plant pathogens is small. It is true also that their number is small in comparison to the fungous pathogens that attack cultivated plants.

The plant pathogens so far reported and confirmed are all rod forms. No cocci are known to exist, although some have been described; but in all such instances the work has been disproved, or the species never again found. The species that infect and produce disease in the growing green plant are nonspore formers. A few spore formers, members of the genus *Bacillus*, exist that can only cause rots in stored tubers, bulbs, etc., but these species are not treated in this review. Furthermore, the true pathogens are strict aerobes to facultative anaerobes, have a simple physiology, and as a rule can readily be isolated and grown in ordinary media. The majority utilize ammonium salts, and only a few require an organic nitrogen source. It is surprising that not one of the species has been shown to attack cellulose, and that the number hydrolyzing starch is small. Their approximate optimum temperature for growth is 27°C., a few higher and some as low as 22°C. Few grow at 37°C., and their minimum temperature approaches 0°C. We have omitted mention of the legume root nodule bacteria which in a sense are phytopathogenic, but they have a literature so voluminous and extensive that they would require an entire review. The actinomycetes also are not discussed here.

The plant pathologist has always had close ties with the mycologists and from this association has been conscious of a taxonomy of fungi based on morphology. Thus, shape of cell, presence or absence and position of flagella in the bacteria, as set forth by Migula, was readily followed. Later Smith's classification based on the same characters but substituting the name *Bacterium* for *Pseudomonas* and *Aplanobacter* for *Bacterium* was adopted. In Europe, both of these systems were used as was the system of

Lehmann and Newmann. With the first edition of *Bergey's Manual of Determinative Bacteriology*, the phytopathogenic bacteria were segregated and placed in two genera, *Erwinia* composed of motile bacteria with peritrichous flagella, and *Phytomonas* composed of nonmotile and polar flagellate forms. The inadequacy of this classification was soon recognized and various groupings in the above genera were demonstrated by Burkholder (11) and Dowson (12, 13). While some recognition was made of these groups in the fifth edition of *Bergey's Manual* it was not until the sixth edition that plant pathogenicity was discarded as a generic character and the species, especially in *Phytomonas*, allocated to those genera where they apparently belong. It is this last system of classification that is used in this review, and only five genera contain the majority of the plant pathogens. Elliott (14) has discussed the trends in recent years in the classifications of the plant pathogens.

Pseudomonas.—A little less than half the phytopathogenic species are placed in the genus *Pseudomonas*. These are gram negative bacteria with polar flagella that are able to grow in simple synthetic media, do not as a rule act upon the higher organic compounds, and are acid sensitive. Many produce a water-soluble green fluorescent pigment, and, while others are nonchromogenic, the major characteristics of all species are similar. Some species are composed of chromogenic and nonchromogenic strains.

Xanthomonas.—The genus *Xanthomonas* contains the second largest group or approximately one fourth of all the plant pathogenic species. This genus was named in 1939 by Dowson (12), but as a group it has been known for some time and its characteristics described (15). It is an interesting genus in that at the present time it contains only plant pathogens. A single polar flagellum is a distinguishing character and the species are acid tolerant. The intracellular yellow pigment occurring in the members of the genus *Xanthomonas* has been shown by Starr (16) to be identical in all species but different from the yellow pigment in species of plant pathogens in other genera. The nutritive requirements also are simple, but certain amino acids are necessary for growth and these vary somewhat with the species (17). On a sugar agar the yellow, mucoid colonies are distinctive and readily detected.

Agrobacterium.—*Agrobacterium* established by Conn (18) in 1942 with *Agr. tumefaciens* as type species also contains the soil bacterium, *Agr. radiobacter*. The genus is related to *Rhizobium*, but its members do not fix nitrogen.

Corynebacterium.—Only nine species of phytopathogenic bacteria have been described as being gram positive. These are closely related to *Corynebacterium* in their morphology, pigmentation, and physiology. At present they have been placed in this genus, although two species are motile, a character that does not exist in the type species *Corynebacterium diphtheriae.*

Erwinia.—In 1920 Winslow *et al.* (19) described a new genus *Erwinia*, the two limiting characters of which are plant pathogenicity and motility with peritrichous flagella. The genus was named in honor of Erwin F. Smith but was never recognized by him. Although based on rather superficial characters the genus does have a certain unity. In keys the species have frequently been divided into two groups on symptomatology: those that produce a blight in plants and those that produce a soft rot. The latter bacteria usually produce acid and sometimes gas from lactose and show relationships to members of the family Enterobacteriaceae. Waldee (20) would split off the soft rot bacteria from *Erwinia* and place them in a genus *Plectobacterium* based on the production of the enzyme protopectinase that dissolves the middle lamella of plant cells.

Bacterium.—*Bacterium stewartii*, a species extensively investigated, does not appear to fit with any degree of exactness into any of the existing genera of the *Eubacteriales.* It is a yellow, nonmotile, gram negative rod, frequently with barred staining, evidence of snapping division, a high salt tolerance, and a complex food requirement. *B. tardicrescens* and *B. albilineans* although motile are similar species.

Distribution of species.—One very interesting point that has come out of the world wide survey of bacterial diseases of plants is the fact that the phytopathogenic species are very similar, and regardless of where they were described they belong to one of the few genera mentioned above. Part of this universal distribution might be due to the dissemination of the pathogens on seed, cuttings, tubers, etc., of our economic plants, which find their way to all parts of the world. This explanation, however, cannot be the entire story, since species of the same genera are not limited to similar climatic areas or even to plants on the same continent. For example, *Xanthomonas citri* occurs on citrus in tropical climates, while *X. taraxaci* produces a disease of the Russian dandelion. *Pseudomonas maculicola* is found on northern grown cauliflower, and *Ps. passiflorae* is found in New Zealand on the passion fruit.

The gram positive vascular parasite of tomatoes, *Corynebacterium michiganense*, was reported first in America; and *C. sepedonicum*, a species on potato, was noted at the same time in Germany. *Erwinia amylovora*, a truly American species, pathogenic on the apple, pear, and related plants, is not found in England; yet a very similar bacterium, *E. salicis*, occurs in England on the willow but has not appeared on this side of the Atlantic. On the other hand, isolated Hawaii appears to have developed its own types of phytopathogenic bacteria. *Xanthomonas rubrilineans*, described first on sugar cane in Hawaii, is a peculiar species; and the bacteria pathogenic on pineapple, isolated and studied by Spiegelberg and Okomoto, and which the writer has examined, are definitely new and peculiar types among the plant pathogens. These pineapple pathogens do have relationships, however, in the Eubacteriales.

Differentiation on basis of pathogenicity.—The differentiation of species in the various genera to which the phytopathogenic bacteria have been allocated has followed the usual procedure in the determination of their morphology, their cultural characters, and their biochemical reactions. In addition to this, pathogenicity also has been used, and in certain of the bacteria it has been the only separating character. An example of such should be cited. *Xanthomonas juglandis* was described by Pierce in 1901 (21) as the cause of the blight of walnuts, and later *X. corylina*, the cause of filbert bacteriosis, was described and named by Miller *et al.* (22). Morphologically, culturally, and biochemically the two species are alike so far as known. Pathogenetically they are distinct entities since they will not cross infect. The use of two names is not only useful but a necessity for the plant pathologists when designating the two pathogens in writing. Other similar cases are on record, especially of those forms in the genera *Pseudomonas* and *Xanthomonas*. In the latter genus there are a number of pathogens culturally similar but differing in the grasses and grains they are able to infect. Hagborg (23) has differentiated these as *forma specialis*, while Wallin (24) has given them varietal names.

Pathogenicity as a specific character must be taken into account in plant pathology since it clarifies many disease relationships among the various crop plants; and where difference in pathogenicity occurs there likely are differences in some biochemical reactions. Burkholder & Starr (25) have shown this to be true in certain pathogens, but the differences in character noted do not

appear to explain the differences in pathogenicity. This same character of pathogenicity also is an aid in identifying a disease-producing organism, and in the use of it the plant pathologist relies less on the regular routine laboratory tests than most bacteriologists. Plants are easily grown in the garden or greenhouse, and numerous inoculations can be made. From the resulting infections, symptoms and suscept range may be studied and the species determined since it is seldom that two pathogens produce identical symptoms. Serological tests have rarely, if ever, been resorted to for determinative purposes.

It has never been successfully demonstrated that plants react with the production of antibodies when inoculated with a specific bacterium. The peculiar anatomy and physiology of the plant probably precludes such a phenomenon, unless the process is cellular and the production of antibodies, therefore, of such limited amounts as not to be measurable. Consequently if serological investigations are conducted with the plant pathogens, it must be done with the standard animal technique. Link (26), an early worker in this field, demonstrated serological difference in many of the phytopathogenic species. That different species react differently is to be expected. *Erwinia amylovora* is a unit serologically according to Elrod (27), while McNew & Braun (28) have shown that *Bacterium stewartii* is made up of various serological strains which have no correlation with other characters studied. *Xanthomonas barbareae* cannot be differentiated with this method from *X. campestris* according to Elrod & Braun (29) although these bacteria produce diseases in different hosts and do not cross-infect; and *X. phaseoli* differs considerably serologically from *X. vignicola*, yet these two species produce the same series of disease symptoms in the common bean (*Phaseolus vulgaris*). *X. vignicola*, however, infects and produces a canker on the cowpea while the former species does not.

Relationships with other bacteria.—The relationships of the phytopathogenic bacteria in the Eubacteriales are with various soil types and are not, with a few exceptions, with the animal pathogens. Probably in the latter group there are no connections even though both animal and plant pathogens are found in *Corynebacterium* and in the actinomycetes. The relationship to the soil types is shown in the striking resemblance, morphologically and physiologically, of the *Pseudomonas* species to the green-fluorescent bac-

teria of the soil. Several species of plant pathogens have been placed in the genus *Agrobacterium* with *Agr. radiobacter;* the soft rot bacteria of the genus *Erwinia* have many characters in common with *Aerobacter aerogenes* and related forms; and Jensen (30) has described several species of *Corynebacterium* that are soil-inhabiting and also related to *C. michiganense* and *C. insidiosum.* On the other hand, the xanthomonads, as far as known, have no counterpart with other bacteria unless it is with *Pseudomonas trifolii.* The yellow saprophytes commonly found on plants are similar in color to species of *Xanthomonas* but those studied by the writer are distinctly unlike in other characters.

This relationship with soil types is evident, and it is reasonable to believe that certain soil bacteria associating with plants over an indefinite period of time may have developed a parasitic nature and become pathogens. It is well known that one may find nonpathogenic bacteria existing within plants in limited numbers, remaining viable and possibly multiplying, but not producing any visible disease symptoms. Perhaps this situation is a step between the saprophyte and the true pathogen. That the process between the two types is a rapid transition is doubted, but it is possibly on a par with other evolutionary trends in the microorganisms. Reid and associates (31) have expressed a belief that the tobacco pathogens known to the plant pathologists as *Pseudomonas tabaci* and *Ps. angulata* are merely the saprophytic soil bacterium *Ps. fluorescens* and that they attack the tobacco under conditions unfavorable to the plant. To support their contentions, data are presented to show that serologically the species are identical. The theory has not been accepted by plant pathologists, especially those working on the tobacco diseases.

If, however, the plant pathogens arose from soil types, they have lost their ability to thrive and multiply in the soil in gaining a parasitic character. *Xanthomonas citri* is an outstanding example of a pathogen's inability to exist without its host plant. Fulton (32) has shown that under soil conditions that would be obtained in agricultural practice, it undergoes a rapid and continuous decline in number. The decline reaches a vanishing point in about two weeks, but it may be retarded by rendering the soil alkaline, lowering temperature, withholding water, or by previously sterilizing the soil. Certain true soil bacteria have been shown to have a marked deleterious effect on *X. citri* in artificial culture media,

and this effect also might occur in nature. Other plant pathogens that have failed to overwinter or remain viable in the soil for long periods are *Corynebacterium michiganense* (33), *C. sepedonicum* (34), *Erwinia amylovora* (35), *E. tracheiplila* (36), *Pseudomonas phaseolicola* (37), *Ps. pisi* (38), *Ps. syringae* (37), *and Xanthomonas juglandis* (39) to name a representative group. Patel (40), however, was able to overwinter eight bacterial pathogens in sterilized and unsterilized soil in test tubes, and it is probable that the length of time the bacteria remain viable in soil depends on the food and the competitors. Valleau and associates (41, 42) have demonstrated that *Pseudomonas tabaci* and *Ps. angulata* from tobacco and *Xanthomonas vesicatoria* from tomato can support themselves on living roots of various plants not considered suscepts and in this manner survive a winter. *Pseudomonas phaseolicola* and *Xanthomonas phaseoli* var. *sojensis* were unable to do this.

Coons & Kotila (43) believe that the bacteriophage plays an important part in the death of *Erwinia atroseptica* in the soil. Since Mallmann (44), Israilsky (45), Anderson (46), Brown & Quirk (47), Chester (48), and Thomas (49) have reported bacteriophages virulent for plant pathogens in water, soil, decaying vegetable matter, and diseased plant tissue but seldom healthy tissue there is probably some relationship. Also the antagonistic action of true soil bacteria towards the plant pathogens as suggested by Lee (50) may aid in their decline under such conditions.

A few plant pathogens appear to have the ability to live for long periods, if not indefinitely, in the soil. *Agrobacterium tumefaciens*, *Erwinia carotovora*, and *Pseudomonas solanacearum* have this reputation. All three, however, have an extensive host range and may be living parasitically on the roots and crowns of various plants instead of in a saprophytic state.

Host and Parasite

The plants subject to one or more bacterial diseases are too numerous to count and tabulate. In 1920, Smith (51) listed 171 genera of flowering plants in which there were at least one species but more often several species that were hosts of a bacterial pathogen. Outside the flowering plants only a conifer, a fern, and a mushroom have been reported subject to a bacterial disease.

Some plant pathogens can infect only one plant or a small group of species in one genus or several related genera. Others have

the ability to infect numerous and unrelated plants. Elliott in her *Manual of Bacterial Plant Pathogens* has listed after the name of each pathogen the plants reported to be susceptible to that species. They vary with the bacterium from one to many dozens of host plants. That certain species have an extensive host range was not recognized at first and on this account several pathogens accumulated a list of synonyms before the situation was clarified. *Pseudomonas syringae* first reported in Holland on lilac by van Hall (52) was later described as a new species on such diverse plants as citrus, cherry, lima bean, cowpea, and hibiscus. The soft rot species of *Erwinia* also have many hosts, and here again there are a number of names undoubtedly that are synonyms. Further work is needed to straighten out this latter group.

There are few of our economic plants, especially among the vegetables, fruits, and field crops, that are not affected by at least one bacterial disease. Among the ornamentals, the percentage of suscepts is not so high, but some of the commonly grown flowers as carnation, delphinium, iris, stocks, and pelargoniums are subject to one or more bacterial diseases. There are also plants that appear to be more susceptible to bacterial diseases than others. The bean and the tomato, for example, are the host plant of four or five different bacterial diseases with pathogens from at least three genera. Many other plants have only one bacterial disease; and buckwheat and spinach, two widely grown crops, appear to be free from bacterial infections. Whether or not these two plants possess an antibiotic substance that the others lack is not known.

Types of infection.—The types of diseases produced in plants by bacteria classified on a symptomological basis are few in number. The most common type is a necrosis that results from local infections and manifests itself in leaf and fruit spots, twig blights, and soft rots of fleshy plant organs. Wilts and stunts due to a vascular infection are fewer in number than the necrotic type but at times are of extreme economic importance. A limited number of bacterial pathogens produce cell stimulation that results in the formation of galls, hairy roots, and fasciations. A chlorosis also is evident in many diseases, but usually it accompanies one of the above types of symptoms and is seldom found alone except for a brief duration. The majority of bacterial diseases readily fall into one of the above groups, but there are certain diseases the result

of an infection by a specific pathogen that may combine several types of symptoms.

The necrotic type of disease, the leaf and fruit spots, are caused by pathogens in the genera *Pseudomonas* and *Xanthomonas*. In these lesions the bacteria are in great numbers in the dead and dying tissue but are not found in the healthy surrounding tissue. A second type of necrosis occurs in the fleshy plant parts, as tubers, bulbs, rhizomes, etc., and is designated as soft rot. *Erwinia carotovora*, *E. atroseptica*, *E. aroideae* and related species are usually involved in this type of disease.

Wilts and stunts are caused, as a rule, by the gram positive bacteria invading the vascular system and filling the xylem vessels with masses of bacteria that extend for considerable distances throughout the plant. These bacteria are with a few exceptions nonmotile and evidently travel with the water movements of the plant. They seldom produce a necrosis to any extent and the walls of the xylem that contain them may be only slightly discolored.

Three other vascular parasites should be mentioned that do not belong to the gram positive group. They produce in monocotyledons blights more than wilts and appear to form a small group bacteriologically and pathologically. They are *Bacterium stewartii*, the cause of the wilt of sweet corn, *B. albilineans* from sugar cane, and *B. tardicrescens*, causing a leaf blight of iris.

A relatively few bacteria affect cell stimulation in plants and produce galls, hairy root, and fasciations. The best known of these diseases is the crown gall caused by *Agrobacterium tumefaciens*. These galls have been known for many years by botanists, but it was not until 1907 that Smith & Townsend proved the cause (53). Since then a vast amount of literature has appeared on the subject. Smith made the investigation of this disease his main work and published during his life time thirty-seven papers (5) concerning it. In most of these papers a comparison is made between crown gall and animal cancer. This comparison has not been accepted by all investigators (54, 55), but at least the galls have been successfully used in the study of pathological stimulation of cell growth. Unlike other bacterial diseases of plants the pathogen is not found in large masses in the gall tissue but occurs intercellularly in relatively small numbers. Since Smith's time Riker (55) contributed greatly to our knowledge of the disease and has writ-

ten excellent reviews on the subject. Of recent importance are the investigations of Braun & White (56) showing that secondary crown galls may be free of the pathogen. Such galls when grown in tissue cultures and again transplanted into the host may continue their growth.

In 1913 Smith (57) reported on a "new type of bacterial disease" that does not fit in with the symptom types just discussed. The bacteria, in yellow slimy masses, occur externally on the heads of grasses and grains evidently smothering them. Later the bacteria may penetrate the host tissue. The bacteria are diphtheroids, and a European species has been described as *Aplanobacter rathayi*. In America a similar or maybe the same bacterium has been named *Bacterium agropyri* and in Asia, *Bacterium tritici*. The three pathogens have been placed in the genus *Corynebacterium* but no comparative study has been made of them. Economically they are of little importance.

Exceptions, of course, are to be found in the above groupings. *Xanthomonas campestris* and *X. phaseoli*, besides producing necrotic lesions, are vascular in the crucifers and in beans, respectively. *Pseudomonas phaseolicola* also becomes vascular in the bean and produces at times a stunt, a wilt, and a chlorosis. Riker *et al.* (58) have listed a number of not too closely related bacterial pathogens that cause a stimulation of the host cells followed by galls or other hypertrophies.

Infection.—The life histories of the phytopathogenic bacteria, in relation to the diseases they produce in plants, while varying somewhat with the species, have certain salient features in common. The amount of inoculum to initiate infection must be rather small under normal conditions in the field. Under controlled conditions Hildebrand (59, 60) has produced infection with single cells of *Erwinia amylovora* and of *Agrobacterium tumefaciens* on pear blossoms and tomato stems, respectively. Whether this holds true with other diseases can not be stated with certainty.

The time between inoculation of a plant and the appearance of the first symptom, or the so-called incubation period, varies greatly with the disease, the environmental conditions, and the state of the host plant. The soft rot bacteria when applied to a potato tuber in a liberal amount produces a definite rot in fifteen hours. Many leaf spots require approximately a week, and a duration of months is necessary before recognizable symptoms of the

alfalfa wilt appear. It also has been held that a large mass of bacteria will shorten the incubation period, but no data are on record. Skaptason (61) found that a large inoculum of *Corynebacterium sepedonicum*, the potato ring rot agent, decreases considerably the time required for colony development on solid media and decreases the lag period in liquid media. *In vivo* the same might be true.

In initiating infection the bacterial plant pathogens gain entrance to the plant either through wounds or through natural openings. The latter may be stomates, water pores, lenticels, or nectaries. The stomates probably serve as the most common portal, at least for the leaf spot organisms, since they are distributed so universally over the leaves. These stomates, however, may not always be open to allow entrance to take place. Varying environmental conditions react upon the guard cells of the stomata causing them to close or open as the case might be and only under the latter condition can the bacteria enter to set up infection. Moisture also is necessary for the bacteria to enter and Clayton (62) has demonstrated that when the leaves are water-soaked internally, as after a rain, infection takes place readily and the disease lesions develop more rapidly than under drier and more normal conditions. Within the stomatal cavity sufficient nutrients occur for the growth and multiplication of the bacteria. Here the pathogens penetrate between the parenchymatous cells of the leaf probably by the aid of a pectic enzyme that dissolves the middle lamella, and in some diseases invade the host cells. In the intercellular spaces some pathogens develop as gummy masses that enlarge and push aside and kill the adjacent cells. One theory advanced by Bachmann (63) holds that the death of the cells results from plasmolysis due to the high osmotic pressure set up in the intercellular spaces by the bacterial invasion. A second theory, and one generally held, is that a toxic substance produced by the bacteria diffuses into and kills the plant cells. It is very evident that in many bacterial leaf spots chlorotic cells may extend for some distance beyond the bacterial invasion and produce varying types of borders or halos about the lesions. *Pseudomonas phaseolicola* causes a very small necrotic lesion on the bean leaf, but it may be surrounded by a band of chlorotic cells an inch wide. A toxic substance would seem to be involved in such a disease. That *Pseudomonas tabaci*, the cause of the wild fire of tobacco, produces a toxin in various media has been demonstrated by Clayton (64).

This toxin when filtered free of the bacteria and introduced into a tobacco leaf by pricking destroys the chlorophyll of the cells and produces the halo symptom of the disease. Furthermore, many and various unrelated plants are sensitive to the toxin, and some, as the bean, are more sensitive than the tobacco. These results throw doubt on many of the listed host plants of various pathogens where inoculations were made by wounding, unless it has been ascertained that the bacteria can establish themselves and invade the healthy tissue.

In a few diseases, although the list is not large, the pathogen, after stomatal infection has taken place, may enter the primary xylem in the leaf and pass in vessels into the stalk of the plant and become systemic. *Xanthomonas phaseoli* and to a greater extent *Pseudomonas physeolicola* may accomplish this in the bean as shown by Burkholder (65). Thus a plant may become infected in the stem and break forth in a necrotic lesion in a leaf. Many water pore lesions on the cabbage and cauliflower caused by *X. campestris* are the results of a systemic infection and the lesions are of endogenous origin.

While the species of *Pseudomonas* and *Xanthomonas* that cause leaf spots, and a few other phytopathogenic species gain entrance to the plant through natural openings, many of the bacterial pathogens require a wound. The pathogens in this class are the vascular invaders, the gram positive species in *Corynebacterium* and the gram negative *Bacterium stewartii*, the gall producer, *Agrobacterium tumefaciens*, and with a few exceptions the soft rot bacteria. Wounds in our economic plants are universal due to handling and cultivation. The transplanting of seedlings and scions, the cultivation, and the trimming all give rise to small or large injuries. *Corynebacterium sepedonicum* gains entrance to the vascular system of the potato seed piece at cutting time and from here passes into the vascular system of the growing plant. *C. insidiosum* infects the alfalfa through winter injuries according to Jones (66), and the cut stems at mowing time. Brown (67) has shown that *Bacterium gypsophilae* infects its host through imperfect grafts and *B. stewartii* enters the corn leaf with the bite of a beetle as demonstrated by Rand & Cash (68). Since all these pathogens establish themselves in the xylem vessels, some species, such as *Corynebacterium flaccumfaceins* and *C. michiganense*, enter the seed and are ready to infect the young plant the following year. *Erwinia*

carotovora, the universal soft rot bacterium, as a rule, requires a wound to enter a fleshy organ; but it also has been shown by Smith & Ramsey (69) to use the lenticels in infecting potato tubers under wet conditions.

After gaining entrance to a plant these vascular parasites proceed slowly between the cells of cortex and invade the xylem vessels or, if the wound is sufficiently deep, they infect the xylem at once. *C. flaccumfaciens*, the bean wilt agent described by Hedges (70), cannot establish itself on the parenchymatous cells of the pod when introduced artificially but growth in the vessels is rapid. Bryan's work (71) on *C. michiganense* establishes the fact that the pathogen in the vascular system of the tomato may break through the xylem vessels, form bacterial pockets in the pith, phloem, and cortex, that result in cankers along the stem.

The soft rot species of *Erwinia* progress rapidly through storage organs of plants as tubers, bulbs, rhizomes, etc., with the aid of an enzyme that dissolves the middle lamella and breaks apart the cells as first shown by Jones (72). In the iris rhizomes and growing underground parts the plant reacts to the disease by laying down a cork layer through which the pathogen cannot penetrate, and the disease is checked unless the cork layer is broken.

At times nonpathogenic bacteria may be found in the lesions aiding or hindering the pathogen in infection. In 1932 Burkholder & Guterman (73) reported the case of a nonpathogenic bacterium that aided *Xanthomonas hederae* in producing lesions on ivy leaves; and White & McCullock (74) found later an anergistic action with nine saprophytic associated bacteria in the same disease. From personal observations the writer is of the opinion that the latter instance is of frequent occurrence among bacterial diseases of plants.

Dissemination and maintenance.—Without a method or methods of dissemination and the ability to exist through the winter in the temperate zone or the dry periods in the tropics, a phytopathogenic bacterium would not survive long. Our common plant pathogens are universal, probably because they have successfully developed the ability to do both. Their survival through unfavorable conditions is usually in association with their host plant or plants where they have attained a dependency that leaves them vulnerable when this association fails. They form no spores nor resting bodies and it has been shown earlier that with the ex-

ception of a few species, their chances of survival in the soil are dubious.

The methods the plant pathogens adopt in overwintering are few and simple. Those that infect trees or woody plants may remain alive in cankers, blighted twigs, or galls, as is the habit of the agents of fire blight, walnut blight, or crown gall. Other pathogens remain viable in living or dead leaves as *X. geranii* (75) or *X. pruni* (76) and the bacteria infecting annuals frequently penetrate the pod and live in or on the seeds of their host. Within the seed they are very definitely given protection, at times as if lyophilized. *Corynebacterium flaccumfaciens* has remained viable according to Burkholder (77) in bean seed for twenty-four years under laboratory conditions. This time exceeds the viability of the host seed by many years. The potato tuber harbors its pathogens *Erwinia atroseptica*, the black-leg agent, and *C. sepedonicum*, the cause of the ring rot and wilt.

At least two pathogens have gone astray and overwinter in a unique but at least an effective manner. *Erwinia traceiphila*, that causes the wilt of cucumbers, overwinters according to Rand & Cash (78) in the striped cucumber beetle and *Bacterium stewartii*, the agent of sweet corn wilt, has been shown by Elliott & Poos (79) to survive the winter in the brassy flea beetle.

Most of these methods of surviving unfavorable periods for the pathogen also aid in the dissemination of the species, since the seeds, tubers, and cuttings that they infect are distributed for propagation purposes and frequently carry their pathogens with them. Once in a locality the bacteria may be spread, at least in a field, by rain, winds, farm implements, animals, and even man.

In the dissemination of pathogens from field to field, insects have been recognized as vectors from very early investigations. The fire blight of apple and pear was a favorable disease for such observations. Hold over cankers on the limbs of infected trees produced bacterial ooze in the spring, and blossom blight occurs through the infection of floral nectaries. That insects were vectors seemed evident and Waite (80) in 1891 infected pear blossoms with the pathogen and allowed bees to transmit the bacteria from blossom to blossom. Gossard (81) incriminated the bee hive as a source of inoculum, but Thomas & Ark (82) are of the opinion that *E. amylovora* can live here for only a short duration. These investigators also list many vectors for the pathogen.

No doubt insects are involved in one way or another in the dissemination of most of the phytopathogenic bacteria, sometimes as carriers and also aiding in direct inoculations as shown by Leach (83) for the pathogen of the black leg of potatoes.

Many bacterial diseases of plants are enphytotic and cause only minor losses from year to year. At times, however, one of these diseases breaks forth and becomes extremely severe. Such a case is that of the sweet corn wilt, observed first some fifty years ago, which has had intermittent outbreaks that became very severe in the early thirties when they destroyed approximately 15 per cent of the crop (84). Stevens (85) pointed out that these epiphytotics of the wilt followed mild winters; but since winter temperature could have no direct effect upon the disease the following summer, Elliott (86) suggested that the effect was on the number and distribution of infested flea beetles. She (87) further supported her theory by demonstrating that the severity of the wilt in 1938 in the northeastern states was correlated with the population of this insect.

On the other hand, weather may have a direct effect upon a bacterial disease. The halo blight of beans is more severe in cool, wet summers according to Burkholder (65) and Goss (88). The hot dry areas of California eliminate the pathogen entirely when beans are grown there. *Pseudomonas solanacearum*, the cause of brown rot of many plants, is favored by high temperatures; and while it is enphytotic in many agricultural sections it is limited to the warm and hot zones.

The sudden appearance of hitherto unreported bacterial diseases that immediately reach epiphytotic form is of interest and at times puzzling. The halo blight of beans caused by *Pseudomonas phaseolicola* was observed first in 1925 (89). It destroyed thousands of acres of susceptible varieties of beans in a few years, and within a decade was reported in most of the bean areas of the world. The dissemination of the pathogen can be explained in that it accompanied the seed in its world-wide distribution, but the origin of the disease is not clear. It should be pointed out, however, that at the time the halo blight appeared in America, a disease of the kudzu vine was reported by Hedges (90) caused by the same pathogen. This legume is of Asiatic import and the growing of it was being encouraged at that time in the southern states of America. It is possible that the halo blight bacterium was imported on

the kudzu vine where it was causing little damage, but once on the bean it became a pathogen of extreme importance.

Virulence.—It is commonly believed that the virulence of a plant pathogen declines and is lost in culture, but is enhanced in passage through its host plant. This phenomenon probably varies with the pathogen. *Erwinia carotovora* A.T.C.C. 495 still produces a rot of fleshy tubers after twenty-five years in culture and *E. phytophthora* (*atroseptica*) A.T.C.C. 496 isolated before 1906 will produce a rot of potato tubers but has lost its ability to infect the growing plant and cause the black leg symptom. Nakata (91) states that *P. solanacearum* retains its virulence

> long, sometimes being rather intensified when cultured in media from natural origin while they lost it quickly in media synthetically made.

Van Lanen *et al.* (92) by successive transfers of virulent cultures of the crown gall bacteria in a dozen amino acids destroyed the virulence of these cultures.

The investigations of Wellhausen (93), McNew (94, 95), and Lincoln (96, 97) on variability in *Bacterium stewartii* throws considerable light on the problem of virulence with this pathogen and it is reasonable to believe that it holds good with other pathogenic bacteria. The discovery was first made that successive passages of a culture of this organism through highly resistant maize plants increased the virulance of the initial strain of *B. stewartii*, but when successive passages were made through a susceptible host, the reverse occurred and the culture became weakly virulent. The virulence could not be increased or decreased beyond a certain point, the parasite appearing to reach an equilibrium with its particular host environment, after which further passages were of no avail. Examination of cultures, single celled and otherwise, demonstrated the fact that they consisted of several strains varying in colony character and color, correlated with virulence. During host passages, a selection is made on these strains, the resistant host selected for multiplication the more virulent strains and the susceptible hosts the less virulent one. The assumption from this work is that the virulence of any culture is a direct function of the proportion of virulent and avirulent organisms present.

The origin of variants in a culture is considered to be through mutation, and the calculated mutation rate of *B. stewartii* under normal conditions ranges from 1 in 20,000 to 1 in 800,000. Both

increases and decreases in virulence have been observed and many variants are as stable as the parent type. X-irradiation of low quantum energy increases the rate of mutation but makes no difference in the type of mutants. Physiological differences have been noted in that the virulent types reduce nitrates to nitrites while the avirulent ones do not. Nitrites, however, are not the sole cause of wilting in this disease.

Control.—The development of control measures for bacterial diseases of plants probably has never been as satisfactory as those for fungous diseases. The applications of sprays that act as a protection against fungi usually do not function as readily with bacterial pathogens, especially with those that become systemic. Eradication of the bacteria on or in seed or from other plant parts, the exclusion of the pathogen from a field or locality by the use of disease free seed stock, and the development and use of disease resistant varieties are methods more satisfactorily employed.

The eradication of bacteria associated with seed has with certain diseases been successful with both heat and chemicals. The immersion of cabbage and cauliflower seed in a hot water bath of 122°F. eliminates *Xanthomonas campestris*, the black rot agent (98). Tons of these seed are treated annually by seed companies and Farm Bureau agencies and where done the disease has been reduced from one of major importance to scattered infected fields. The use of sulfuric acid in delinting cotton seed gives good control of *X. malvacearum* the cause of the angular leaf spot according to Bain (99). Treated seed should, however, in either case not be sown in infested soil.

Antibiotics have so far not been recommended for the control of bacterial diseases but investigations are in progress. Waksman *et al.* (100) tested nine different plant pathogens from five genera as to their sensitivity to six different antibiotics. He also included animal pathogens and saprophytes in his experiments. The conclusions were that phytopathogenic bacteria do not behave as a group but vary in their reactions as is the rule with other bacteria. Brown & Boyle (101) state that penicillin suppresses the growth of *Agrobacterium tumefaciens* and *Erwinia carnegieana*, the sensitivity being on a par with *Staphyloccoccus aureus*. Young crown galls were killed when punctured and crude penicillin applied. Rudolph (102) tested the antibiotic properties of penicillin against

the gram negative *E. amylovora* and *X. juglandis* and found that the substance *in vitro* was not only bacteriostatic but bacteriocidal as well, the latter pathogen being the more sensitive. Attempts to control either organism *in vivo*, however, met with failure.

The eradication of sources of infection as the removal and destruction of hold-over cankers of fire blight is a common practice in orchards. The largest operation on record, however, of an eradication of a bacterial disease was conducted between the years 1914 and 1927 by the State Plant Board of Florida in co-operation with the Bureau of Plant Industry of the U. S. Department of Agriculture (103). The disease under consideration was the citrus canker caused by *Xanthomonas citri* and during the thirteen-year period, 15,243 grove trees and 342,260 nursery trees were destroyed by burning them with an oil spray where they stood. The last infected tree was found in 1927 but inspection was continued for several years afterwards. The disease has not been reported since.

A simple method of disease control is the exclusion of a bacterial pathogen from a field or planting through the use of disease free seeds or cuttings. Several instances might be cited. Beans grown in California in the hot dry sections are free from the bacterial blights, especially the halo blight that is so severe in most other bean sections. California now produces beans largely for seed purposes and much of the Red Kidney beans planted in New York State comes from California. Crops in susceptible areas from such seed show no infection the first year or only slight infections that come in from nearby fields.

The bacterial ring rot and wilt of the potato is a serious disease of this crop and one that so far can be controlled only through exclusion of the disease from the field. Inspection and certification of potatoes for seed purposes under agencies usually connected with the state is conducted in many places. A zero tolerance for ring rot is the standard in certified seed. Thus, with such seed a grower may produce potatoes free from the disease if sufficient care is taken against outside contamination.

One satisfactory method of control of a bacterial disease is through the use of disease resistant varieties. Among varieties, races or single individuals of a commercial crop, resistance or even immunity is frequently found. If the resistant types are of good, desirable quality they are grown forthwith to combat the disease

in question, but if the resistant type has certain undesirable characters they are saved to hybridize with commonly grown economic varieties. This is a common method to combat fungous diseases, but also has been used successfully in certain bacterial diseases where other methods of control have failed.

Susceptible and resistant varieties of sweet corn to Stewart's disease had been known for some time but not until the severe epiphytotics of this disease during the thirties was use made of the resistance. Wellhausen (104) presented data showing that resistance in corn to the wilt was governed by at least three (2 major and one minor) dominant, independently inherited supplementary factors. From this work the breeding of resistant varieties could proceed. At present many Agricultural Experiment Stations and seed companies are producing through hybridization new sweet corn varieties with both resistance to the disease and desirable commercial characters. Dent corn is also a host to *Bacterium stewartii* and although it does not suffer the severe losses that sweet corn does the disease is of importance. Elliott (105) has shown in certain inbred lines of dent a peculiar type of resistance that should be taken into account in any breeding program. Certain lines are resistant in the seedling stage but become susceptible at maturity and in other lines the reverse is true.

The wilt of alfalfa first recognized in 1924 but now widespread in the United States is a plant disease of major importance. At one time alfalfa could be planted and left down almost any number of years. Now, three and four year stands are killed out entirely and the wilt destroys hundreds of thousands of acres annually. The nature of the crop and of the disease makes the use of resistant varieties the only feasible method of control. Fortunately, Peltier (106) and Jones (107) in tests of alfalfa varieties gathered from Europe, Asia, Africa, and the Americas demonstrated that seed from Trukestan and Ladak gave plants of a high degree of resistance. Wilson (108) has found the character of resistance to be inherited; it involves three or four partially dominant genes and can be introduced into the commonly grown commerical varieties.

Knight & Clousten (109) have found resistance and complete immunity to the black arm of cotton caused by *Xanthomonas malvacearum*. Resistance is inherited (two factors are involved) and can be transferred to other varieties without disturbing desired qualities in the cotton.

Investigations of the bacterial blights of beans have demonstrated that resistance to one bacterial disease is not identical or even correlated to resistance to another bacterial disease even though the two pathogens are similar in the type of symptoms they produce. Burkholder & Bullard (110) have shown that the Hidatsa Red, a little grown variety of beans, immune to the halo blight caused by *Pseudomonas phaseolicola*, is very susceptible to the fuscous blight caused by *Xanthomonas phaseoli* var. *fuscans*. Also the Peerless variety is resistant to the halo blight but susceptible to the common blight caused by *X. phaseoli*. Thus it is seldom that a variety of plants is produced resistant to all diseases. For this reason a grower is forced to resort to combinations of various disease control methods, together with good cultural practices to insure healthy plants. Furthermore the methods used will vary with climatic and soil conditions under which the plants are grown.

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CHEMICAL DISINFECTANTS[1]

By Orville Wyss

Department of Bacteriology
The University of Texas, Austin, Texas

Chemical disinfectants in the customary sense are those agents which are used primarily to destroy microorganisms and not merely to arrest their growth. To some the term "disinfect" implies the destruction of pathogenic organisms but most workers include the destruction, as for example in the dairy industry, of any organisms whose continued existence would result in undesirable consequences. The lack of a sharp line of demarkation between disinfectants and the antiseptics or bacteriostatic agents is well recognized. In a treatise on injury and death of bacteria by chemical agents Rahn (1) found it convenient to regard as disinfectants those substances which would, if tested by the common phenol coefficient procedure, yield sterile transfer tubes after one-hour exposure to the most concentrated of the commonly used solutions. Obviously many agents cannot qualify under these conditions. Some, such as the arsenicals and quinine, whose action is primarily lethal for the specific organisms against which they are employed, cannot be subjected to the test for technical reasons. Many of these substances which are of current interest will not be considered in this chapter since they are reviewed in the chapters of this volume on chemotherapeutic agents and antibiotics. The food preservatives, which have been reviewed recently by Wyss (2), and other compounds whose action is primarily bacteriostatic will not be included.

The most obvious trend in the literature on chemical disinfectants in the last ten years has resulted from the decline in the use and the promotion for use of disinfectants for wound treatment. The advent of sulfonamides and antibiotics for the control of systemic infections and the appreciation that such bacteriostatic agents, with the aid of the natural tissue defenses, constitute the most effective treatment for the elimination of localized infections is now almost universally accepted. Although the clinical literature is not entirely consistent, a considerable number of clinicians appear to agree with Shambaugh (3) that treatment of contaminated

[1] This review covers the literature up to February, 1948.

wounds with disinfectants (alcohol, iodine, mercurials, quaternary ammonium compounds) favors rather than inhibits the development of suppurative infection. As a result, certain experimental approaches which were regarded as the best practical methods of evaluation, such as those showing the effect of pus and blood on disinfectant action, have been largely abandoned. The problem of tissue toxicity, excepting as a measure of irritation to skin and mucous membrane, has become one of minor importance. The search for disinfectants selectively absorbed by bacterial protoplasm as opposed to the protoplasm of the host tissue is generally conceded as a lost cause, especially in view of the successful application of agents which selectively inhibit the metabolic activities of the bacterial cell.

This does not imply that the study of disinfectants and disinfectant action is no longer of interest to the microbiologist. Large quantities of germicidal agents are used for preoperative disinfection of the skin and for reducing or modifying the flora of the mucous membrane of the mouth, the nasopharynx, and the urogenital tract although the usefulness of some of these applications has been disputed. Some disinfectants, for example, iodine, are found effective for surface application to such infective processes as boils not because of their germicidal action but because of their counterirritant stimulation of tissue activity. In the treatment of superficial infections of the skin and mucous membranes disinfectants are employed routinely, though in certain pathogenic processes antiseptic substances are regarded as superior therapeutic agents because of their milder action. In the destruction of microorganisms in the nonliving environment the disinfectants have maintained their interest for the microbiologist, and new fields of action such as aerial disinfection are developing. In addition, the biologist interested in the fundamental principles of death finds an effective tool in the application of disinfectants.

Methods

The methods for evaluating disinfectant action have been subjected to critical study. Such methods fall roughly into three groups. On one extreme, experiments are devised to measure the effect of individual factors on the activity of the disinfectant and by variation of these factors to obtain sufficient information to enable us to predict the performance of a disinfectant under any

condition of application. This scientific approach has been applied successfully by Charlton & Levine (4) for the study of the disinfectant action of chlorine. The test organism was a standardized spore suspension of *Bacillus metiens*, which exhibited practically no variation in resistance throughout the series of tests. The menstruum was a buffered solution of the disinfectant in a large flask equipped with a stirrer and placed in a water bath, thus eliminating minor environmental variations. At measured time intervals samples from the mixture of organisms and disinfectant were removed to tubes containing an antidote for disinfectant action and plate counts were made to determine the number of survivors. As an end point the time for 99 per cent killing was used, a point which was determined from a graph of the log of the number of survivors plotted against time. This point was on the straight line portion of the killing curve and was determined by three or more plate count data obtained for the several time intervals. The effects on germicidal action of variations in the concentration of the disinfectant, the temperature, and the pH were determined by this method. The interference with disinfectant action of specific chemical groupings was determined by the addition to the medium of chemical reagents containing the active group which might be implicated in an effect on the germicidal action. For example, since the action of chlorine is affected by the presence of nitrogenous compounds, ammonia and various amines were added to the buffered test solution to determine their influence on the lethal action. In this manner the composite picture of the action of the disinfectant was obtained.

Withell (5) recommended a similar method for evaluating disinfectants except that he used nonclumping vegetative cells washed from agar slants. Survivors were determined by plate counts and the percentage response of the test organism to each disinfectant was converted to probits and plotted against a logarithmic time scale. From the resulting straight lines obtained with various disinfectants the lethal times for 50 per cent response were compared to determine (*a*) the relative speeds of action of two disinfectants at any convenient concentration; (*b*) the concentration coefficients; and (*c*) the temperature coefficients. From these observations relative efficiencies of bactericides were calculated.

On the other extreme are those experiments designed to test the effectiveness of the disinfectant under conditions simulating

as nearly as possible those under which the agent actually will be put to use. Gardner (6) prepared infected blood clots and determined the effectiveness of disinfectants in sterilizing them under standard conditions. Gee & Sarles (7) infected trout eggs with the organism causing furunculosis and evaluated the efficiency of various disinfectants in destroying the organisms without affecting the viability of the eggs. Habs & Kirschner (8) tested the efficacy of cutaneous disinfectants on artificially infected guinea pig skin. Sarber (9), Nungester & Kempf (10), Spaulding & Bondi (11), and Pierce & Tilden (12) applied virulent organisms to the skin of a mouse and after treatment with disinfectants excised the skin and inserted it into the peritoneal cavity. Ineffective germicidal action resulted in the death of the mouse. Handwashing techniques as developed by Price (13) and modified by Stuart & Pohle (14), Bernstein (15), and Cromwell & Leffler (16) were used to determine the effectiveness of disinfectants in destroying the bacterial flora of the hands and arms. Gershenfeld & Witlin (17) devised a technique which tested the ability of the disinfectant to prevent or cure infections induced in chick embryos. Mallman, Kivela & Turner (18) introduced a "speed reaction test" involving the sterilization of infected glass rods to determine the efficiency of disinfectants as sanitizers of beverage glasses. These tests yield important and useful information on the efficiency of disinfectants. Yet because of the multiplicity of unknown factors which are operative they yield little information on the general problem of disinfection and the mechanisms by which germicides act.

Between these extremes one might place the phenol coefficient test which, according to some, yields little information of either practical or theoretical importance. That this is not a true evaluation of the test is evidenced by its wide and continued use wherever a simple method for obtaining a relative activity is required. As further support for the test is the fact that although its every aspect has been subjected to extensive scrutiny only minor changes in the procedures have been generally accepted. Rahn (19) and others (20, 21) have discussed proposed changes for the evaluation of disinfectants defending the accepted procedures in certain stages and suggesting that alternative procedures would be desirable in others.

The test requires that the organism have a uniform resistance to phenol, and it has been found that by employing a culture

medium of standard composition and by using standard strains this usually can be accomplished. However, the occasional divergence of the organism from the accepted resistance to phenol causes considerable inconvenience and clearly requires a remedy. The problem is more serious when one considers that the organism's resistance to other chemicals undoubtedly varies independently of its resistance to phenol. Therefore the acceptance of a test as satisfactory merely because the phenol tolerance of the organism is within the accepted limits does not guarantee reproducibility. Grubb & Edwards (22) observed that culturing the test organism at 40°C. will restore to normal phenol tolerance some strains of the organism which have been exhibiting a decreased resistance to that agent.

Wolf (23) reported that nine substrains of *Staphylococcus aureus* strain 209 obtained from different laboratories showed wide variations in their resistance to phenol. He suggested that some one central laboratory maintain the stock strain and have it available for all to use. It has been accepted that media prepared from different brands of peptone will cause variation in resistance of the organisms grown therein. Brewer (24) has shown that the use of different lots of peptone of the same brand caused as much as 100 per cent variation in the phenol coefficient values, indicating less uniformity than one might hope to find in a reputedly standardized product. Such divergent results must be due primarily to variation in (*a*) the amount and nature of the organic matter present; (*b*) the amount of materials which serve as detoxicants for the disinfectant; (*c*) the rate of growth and therefore the physiologic age of the culture at the time of test; and (*d*) the total crop of viable organisms at the time of test. The 0.5 ml. of the broth culture added to the medication tube of the phenol coefficient test contributes about 1500 p.p.m. organic matter. If some is in the form of large molecules considerable adsorption of certain types of disinfectants will result. This factor is dependent on the extent of the hydrolysis undergone by the peptone in its preparation. Certain reactive groups of the proteinaceous material such as the hydroxyl, carboxyl, amino, sulfhydryl, and disulfide groups may react with disinfectants and affect their activity and the numbers and nature of these groups may vary in different lots of peptone. By reacting with the disinfectants they change the effectiveness of the chemicals or neutralize their action completely. Other detoxi-

cants may be present such as phospholipids which have been shown by Baker, Harrison & Miller (25) to inactivate quaternary ammonium compounds. Even such simple materials as the chloride ion will inactivate some germicides; the phenol coefficient of silver nitrate is determined by the sum of the amount required to precipitate out the chloride ion present in the medium plus that required to destroy the organism. Minute amounts of iodine or chlorine are generally required to kill microorganisms but reducing substances in the broth almost instantly destroy a portion of the amount added. Until this demand is satisfied none of the disinfectant is available for killing microorganisms. An illustration of this point is evident in the data of Salle & Catlin (26) from a modified phenol coefficient experiment. Fifty parts per million iodine (one to 20,000) killed the organisms in the ten minutes allotted by the phenol coefficient test but when the medication tube was sampled for living organisms at shorter intervals it was found that complete killing actually occurred after only one minute exposure. The next tube which contained 47.7 p.p.m. iodine did not kill all the organisms even in ten minutes. From our knowledge of the concentration coefficient of iodine we can conclude that about 47.7 p.p.m. of iodine were destroyed by the reducing action of the broth and that the organisms were killed in less than a minute by 2.3 p.p.m. iodine. It appears to the author that this typifies the greatest weakness of the phenol coefficient test and he believes that from both practical and theoretical considerations the effect of various types of organic matter might better be determined separately to fit the intended application.

For greater uniformity in the phenol coefficient test Wolf (23) suggested a casein hydrolysate medium for culturing the organism. Klarmann & Wright (27) prepared a semisynthetic medium for the purpose of eliminating one of the variables in the phenol coefficient test, namely, the nonuniformity of peptone. Such media differ from the phenol coefficient medium in the nature and amount of organic matter and would affect the result obtained with some disinfectants more than with others. The use of peptone, blood, or milk in disinfectant testing to determine activity in the presence of "natural" materials may be misleading. McIlwain (28) points out the fallacy with the example of the sulfonamides with which a synthetic medium generally simulates more closely the conditions governing *in vivo* effectiveness than do any of the so-called "natural" media. The results obtained in any medium give the relative

activities of related compounds but cannot be interpreted too broadly.

No appreciable differences in the results observed in various laboratories doing phenol coefficient tests would be expected from variation in the number of organisms added to the medication tube. Extreme variations of the original culture density such as from five hundred million to two billion organisms per ml. will change the killing time less than 10 per cent (19). Only when the inoculum size is varied from ten- to one hundred fold will there be a detectable change in the end result of the phenol coefficient test. Similarly one would expect little effect from small variations in the size of the sample which is removed from the medication tube to the subculture tube except from the point of view of carrying over toxic materials. Yet a significant change in the phenol coefficient of a synthetic detergent was observed by Tobie & Orr (29) when a pipette was substituted for the standard loop. They found that from medication tubes containing surface active agents the standard loop picked up much less than the expected .01 ml. The subculturing of the entire medication tube will give a different result from the use of loop dilutions to a second subculture tube. In the latter case negative culture tubes do not imply that all organisms in the medication tube were killed but merely imply that enough of them were killed so that none was present in the sample which was transferred to the subculture medium.

With most disinfectants antidotes are required to stop the killing action at the time of sampling. For substances with high concentration coefficients such as phenol it was generally believed that dilution in the culture medium was adequate for neutralizing the germicidal action. However, Flett and co-workers (30) showed that the addition of activated carbon or ferric chloride to the bacteria after exposure to the disinfectant "revived" many that otherwise would have failed to grow. The concentration of phenol which "killed" in ten minutes required twenty-five minutes when the colloids were added to the subculture medium. The carbon or iron colloid adsorbed the phenol away from the bacterial cells and prevented the continuation of the lethal action after the measured exposure time.

Valko & Du Bois (31) demonstrated that after exposure of cells to surface active cations for sufficient time so that subcultures in broth would show no survivors one could demonstrate that some cells were still alive by treating the organisms with a high molecu-

lar weight anion. Apparently in the initial step of the killing process the cationic substances are adsorbed by the bacterial cell. If they are not removed their subsequent action results in cell death. Dilution in the subculture medium does not quickly and effectively bring about desorption but this can be accomplished by the addition of a high molecular weight anion.

Mercapto compounds are rapid antidotes for the killing action of heavy metals. The concentration coefficient of these compounds is so small that even extreme dilution, such as the removal of a few loopsful from the first subculture tube to a second tube as recommended by Shippen (32), fails to alleviate the static action but the addition of a sulfide or mercapto compound to the first subculture tube readily neutralizes the activity. Marshall, Gunnison & Luxen (33) used thioglycollate medium for subculturing organisms exposed to mercurials. Since the killing action of mercury was relatively slow the use of thioglycollate media subculture gave results that were very different from those obtained with plain broth or even in broth enriched with blood or other organic supplements.

With many disinfectants a lower activity is obtained when an enriched broth is used for subculture. Several explanations present themselves: (*a*) the richer broth is a better antidote for the disinfectant; (*b*) the richer broth permits growth from a smaller inoculum; (*c*) the injured cells are more exacting in their nutritional requirements; (*d*) colloidal materials in the enrichment adsorb toxic materials from the broth which are inhibitory to the injured cells. All the factors may be operative but the work on recovery of heat injured spores by Curran & Evans (34) suggests that the last explanation may play an important role. These workers found that the addition of starch to media rendered it suitable for prompt growth of heat injured spores which otherwise failed to develop or developed only after an extended dormancy period. Pollock's successful replacement of blood with charcoal in the media for organisms which were formerly thought to be fastidious in their nutritional requirements (35) emphasizes the necessity of removing toxic factors from the medium. These toxic factors generally are fatty acids which are surface active compounds. In view of the synergistic effects of wetting agents and phenol observed by Ordal, Wilson & Borg (36) it appears reasonable that some of the detoxifying effect of charcoal in subcultures may be due to the alleviation of the toxic action of the subculture medium. One anticipates that

such toxic action would be more evident when the inoculum has been exposed to disinfectant action.

In interpreting results of tests done by the phenol coefficient method one must not fall into the surprisingly common error of regarding the culture in the medication tube as an individual which is either killed or not killed as indicated by the subculture tube. Even such an outstanding authority on the dynamics of death as Rahn is guilty of statements which could mislead a casual reader such as his comment (19) that "H_2S can restore the power of reproduction to staphylococci after 16 but not after 33 hours' exposure to mercuric chloride." He states further

> Valko *et al.* treated bacteria "sterilized" by cationic detergents with anionic detergents, and if this were done within thirty minutes after exposure, the bacteria became capable of reproduction.

Presumably death occurs as some progressive function of time in spite of the addition of the antidote at the time of sampling, but some organisms survive a much longer exposure period than is the case if no antidote is used.

Surface Active Agents

The surface active agents have been reviewed adequately by Rahn & van Eseltine (37). Additional evidence has appeared in support of Hotchkiss' explanation (38) of the death of cells as being due to an effect by these chemicals on the semipermeable nature of the cell membrane. Gale & Taylor (39) showed that glutamic acid and lysine from the internal cell invironment of bacteria and yeast rapidly leaked out of cells treated with detergents. This did not occur after the application of nonsurface active disinfectants but did occur with both anionic and cationic wetting agents. Mitchell & Crowe (40) presented electron photomicrographs showing the disrupted cell wall in streptococci treated with detergents. Presumably the cell wall is the affected confining barrier or perhaps it is attached to the cell membrane and breaks with it.

Mercurials and Other Heavy Metals

The mercurials have been as much maligned during the past few years as they had been exalted in previous decades. The low phenol coefficient values obtained when effective antidotes are supplied have been rather surprising, but we are slowly coming to the appreciation that the phenol coefficients of mercurials are in the

neighborhood of one instead of approximately one hundred to two hundred as is the case if no antidotes are used in the subculture medium. The slow action of mercury is due to a number of factors Salle & Ginoza (41), in a study of the effect of the medium constituents on the germicidal action of mercuric chloride, found that such organic compounds as sugars had no adverse effect. On the other hand equimolar solutions of the amino acids interfered with the killing as did gelatin, peptone, and peptides. The interference was proportional to the free amino groups. Cysteine greatly inhibited germicidal action but such compounds are not present in significant amounts in nutrient broth. Fildes (42) was unable to observe any interference with mercurial disinfection by the amino acids tryptophane, cystine, or methionine, or by any of the vitamins but compounds containing sulfhydryl groups markedly interfered with the germicidal action. By chemical test the mercury atom was shown to combine with two sulfhydryl groups; and since ordinary culture media contained a negligible number of such sulfhydryl groups, the bacteriostasis can be accomplished by that small amount of mercurial required to tie up the sulfhydryl groups in the inoculum. Actually the affinity of the mercury for the sulfhydryl group varied with the nature of the sulfhydryl compound. A small excess of glutathione (three to four moles per mole of mercury) permitted normal growth in the presence of the mercurial. Thioacetic acid combined with mercury with considerably less avidity and about twenty-five times as much was required to neutralize mercurial action.

From the work of McCalla (43), Gegenbauer (44), and others we can be certain that mercurials are adsorbed on the cells in a reversible manner and that this first step does not damage the cell beyond repair, since at suboptimal concentrations the disinfectants can be removed from a large fraction of the cells and the cell processes proceed normally. Schuler (45) has shown that the cell by itself can muster certain defenses against mercurials or bring about a considerable amount of repair. The respiration of *S. aureus* cell suspensions, inhibited by the limiting amounts of mercurials, would return to the normal rate after standing for a time even though no detoxicants were added. However, in higher concentrations there began immediately secondary reactions which were not reversible and resulted in the death of the cell. The nature of these reactions is not clearly understood but presumably the cell dies from lack of functioning of an active protein. From the dynamics

of disinfectant action it appears that this active protein whose union with mercury results in death is not one of the enzymes of the cytoplasm but rather some essential molecule of the genetic system. Whether this molecule, the gene, is itself an enzyme or merely a pattern for its formation is immaterial; it is a protein and reacts as such with mercury compounds. A defense mechanism involving reduced compounds may explain Grumbach's observation (46) that mercurials appear less effective if subcultures are incubated anaerobically although he suggested that the result indicated a specific poisoning of cytochrome oxidase.

There have been several attempts to relate the amount of mercurial adsorbed with the killing power. Evans & Fishburn (47) found that for any given concentration of mercurial the amount adsorbed on charcoal was proportional to the increase in bactericidal power as measured by the Rideal-Walker method. The almost linear increase with concentration in both functions (charcoal adsorption and bactericidal power) was far less convincing than if there were corresponding maxima or minima in the two curves. In other words, it is possible to find a number of other instances where linear relations exist between disinfectant action and some physical or chemical function. Such data cannot be accepted as more than suggestive of a critical mechanism. Similarly de Lonreiro & Lito (48), who found a quantitative relationship between the thiol content of bacterial suspensions and the amount of mercurial required for sterilization, might have found a similar quantitative relation between this latter statistic and the weight or numbers or the nitrogen content of the cells.

Morton, North & Engley (49) reviewed the pertinent literature on the disinfectant action of mercurials and presented a number of innovations in mercurial testing. The organisms (hemolytic streptococci) were cultured in blood broth and after exposure to the mercurial they were subcultured in thioglycollate medium since blood broth failed to neutralize the bacteriostatic action. The highest concentrations of disinfectants tested (2 per cent mercurochrome, 0.2 per cent metaphen, and 0.1 per cent merthiolate) failed to yield negative subcultures in thioglycollate broth even after fifteen minutes' exposure at room temperature. The possibility was considered that the thioglycollate might have the ability to revive organisms "killed" by mercurials which could not be revived by subculture in living tissues. To determine if the organisms exposed to mercurials still had sufficient vitality to kill mice even

though they failed to grow in the blood broth subcultures some of the mercurial-organism mixture was injected intraperitoneally into white mice. In every test involving mercurials some of the mice died, although in those phenol controls where the thioglycollate subculture tubes were sterile all mice survived. In fact, even in the highest concentration of phenol which gave positive subcultures all mice survived indicating that the subculture tube contained better antidotes for phenol action than did the mice although the mice contained adequate antidotes for mercurial action. Such experiments suggest that we have overrated the disinfectant action of the mercurials, yet from them one can conclude only that when large numbers of hemolytic streptococci are exposed to the commonly used concentrations of mercurial disinfectants in a solution containing 10 per cent blood at least a few organisms survive after fifteen minutes' exposure and these organisms are capable of producing infection. The dynamics of the germicidal action of mercury must be restudied using proper subculture media. However, one can assume that the logarithmic order of death will be approximated and such studies may still show the mercurials to give a margin of safety in their recommended applications equal to that which we accept without question in many of our activities.

Rhodes (50) has employed an ingenious mathematical approach to investigate the mode of fungicidal action of heavy metals. He suggested that when a compound per se can penetrate the cell wall and bring about a lethal reaction the variability of resistance presented by the organisms may be small. If the disinfectant reacts with an excretion product of the spore at the cell surface before penetration, and the lethal reaction arises from the resulting compound, the observed variability of resistance will be greater. He postulated that the greater the number of such successive steps the higher the variability. From experimental data and this postulation it was concluded that mercury and silver thiourea complexes require decomposition before penetration into the cell while copper and zinc thiourea complexes are in themselves the lethal reagents or at least are few steps removed from the actual lethal species.

Chlorine, Iodine, and Bromine

When chlorine is added to water at pH values above 2.0, it hydrolyzes almost completely to give hypochlorous acid and hydrochloric acid of which the former is the active germicidal compound. The hypochlorous acid dissociates according to the ioniza-

tion equilibrium to give the hypochlorite ion which is essentially inactive. Although the dissociation constant of about 10^{-10} for 25°C. has been confirmed by Morris (51) most of the published data on the effect of pH on germicidal action are in closer agreement with an earlier dissociation constant of 10^{-8}. Increase in the hydrogen ion concentration to approximately pH 6.0 increases the activity in proportion to the hypochlorous acid concentrations at equilibrium. Some further slight increase in germicidal activity below pH 6.0 suggests that Cl_2 may have a greater activity than hypochlorous acid.

The "chlorine demand" of the solution to be treated is a measure of the amount of reducing material present which will combine irreversibly with the chlorine to convert it to the inactive chloride and this must be satisfied before appreciable bactericidal action can be obtained. A special type of chlorine demand results from the presence of small amounts of ammonia. If one adds to solutions containing trace amounts of ammonia more than about eight times as much chlorine, the ammonia disappears along with three moles of chlorine for each two moles of ammonia. The end products are volatile nitrogen trichloride or nitrogen gas and chloride ion. The chlorine which persists after this reaction has gone to completion is in the "free" state (i.e., it is not chloramine). The removal of ammonia by excess chlorine and the formation of a free chlorine residual is the basis of the "breakpoint" chlorination process in water treatment (52, 53). This reaction must be considered in any disinfection experiment involving chlorine if ammonia is present in the test solution. The presence of other nitrogen compounds or of excess ammonia (ammonia-chlorine ratio greater than 1:4) results in the formation of chloramines (N-chloro compounds in which the chlorine is still capable of being titrated by reducing compounds but in which state the killing power may be considerably altered). The germicidal action of chloramine was thought to be due entirely to the hypochlorous acid which results from its hydrolysis in water solution but several experimental approaches have demonstrated that the unhydrolyzed chloramine is also a disinfectant. Weber & Levine (54) showed that at pH 10 ammonia-chloramine becomes more active than chlorine itself; this would not occur if the activity were due solely to hypochlorous acid. A change in temperature of 10°C. affected the killing action of the chloramine about twice as much as that of chlorine, although admittedly this temperature effect is a dual one involving both the

hydrolysis equilibrium and the actual germicidal action. The shapes of the curves (log of survivors *vs.* time) were markedly different, with the chloramine giving the standard log death curve but free chlorine exhibiting a marked lag followed by a progressively increasing rate of death. Rahn (1) regarded the lag as evidence that the highly active chlorine unites with many molecules in the cell surface before death results.

Marks, Wyss & Strandskov (55) used a different approach to study the action of N-chloro compounds. Experimenting with a number of substituted amines and amides which were capable of forming only monochloro derivatives they observed an effect on germicidal action by varying the concentration of the nitrogen compound while keeping the chlorine concentration constant. At equimolar ratio of chlorine and nitrogen compound the killing was due to the sum of the action of the hypochlorous acid formed by hydrolysis and the action of the intact molecule. Increasing the concentration of the nitrogen compound shifted the hydrolysis equilibrium to the left thus decreasing the amount of hypochlorous acid and therefore the disinfectant action until a point was reached where the addition of more of the nitrogen compound had no further effect. The killing action at that point was ascribed to the unhydrolyzed N-chloro compound. Such activity varied tremendously with different nitrogen compounds. When no excess nitrogen was present the activity was largely that of the hypochlorous acid and hence was determined by the hydrolysis constant, a value which also varied markedly with different chloramines and chloramides. Since most nitrogen compounds capable of forming N-chloro derivatives were either acids or bases the extent of hydrolysis varied with pH. Compounds such as chloramine-T and halozone became more active with increased pH due to greater hydrolysis to hypochlorous acid. However, as the pH increased, more of the hypochlorous acid ionized to the inactive hypochlorite ion; so as a result of the combined effects these compounds had a maximum activity at about pH 7.0. The germicidal action of chloramines as measured against vegetative cells gave a similar picture to that obtained with spores but the latter were several hundred times more resistant.

Butterfield & Wattie (56) observed that it took about one hundred times as long to obtain equivalent killing with ammonia-chloramine as with "free" chlorine. This figure varied with environmental conditions. At pH values above 8.5, *Escherichia coli*

was more resistant to "free" chlorine than was *Eberthella typhosa;* at pH values below 7.8 the resistances were reversed (57). When chloramine was used as the killing agent the organisms were about equally sensitive. An extensive study on the influence of pH and temperature on the survival of coliforms and enteric pathogens when exposed to "free" chlorine was reported by Butterfield *et al.* (58). Changes in the environmental factors affected the killing of vegetative cells in a manner analagous to that found by Levine and co-workers (4, 54) with spores. The different genera varied only slightly in resistance but under certain conditions of pH, temperature, and concentration the pathogens were more resistant than the coliforms while under other conditions these resistances were reversed.

Ridenour & Ingols (59) found that 0.2 p.p.m. "free" chlorine inactivated a 1:500 dilution of the polio virus in ten minutes while 0.1 p.p.m. required thirty minutes' contact. The same amount was required to kill *E. coli* in a similar time period indicating that for water treated with "free" chlorine the presumptive test for coliforms is a satisfactory method for determining the possible survival of the virus. Apparently the viruses are also much more sensitive to "free" chlorine than to chloramine since Kessel *et al.* (60) observed that a 0.5 p.p.m. chlorine residual (presumably chloramine) required three hours to inactivate the Le strain of the polio virus while an ozone residual of 0.05 to 0.45 p.p.m. destroyed the virus in less than two minutes. Slow action of chloramines against viruses have been observed by others (61, 62). Edwards & Lidwell (63) found that one volume of hypochlorous acid gas in two million volumes of air was sufficient for 99 per cent inactivation of aerosols of the influenza virus.

Chang & Fair (64) using cysts of *Entamoeba histolytica* found that the killing effect of chlorine followed the same general pattern in regard to temperature, pH, and nitrogen as had been observed by others with bacteria. Under the conditions of their experiments *E. coli* was always killed more quickly than the cysts. Chang (65) observed that cysts killed by chlorine showed nuclear disruption and that chlorine could be detected inside the cells by colorimetric tests. The penetrating power of gaseous chlorine was reported to be superior to hypochlorite although it is difficult to visualize any difference in aqueous solutions of these substances when at equilibrium.

Chlorine dioxide has been tried as a substitute for chlorine in

water purification and is reported to exhibit certain advantages under some conditions. Synan *et al.* (66) found it superior to chlorine in the elimination of coliforms from pure water but because of its greater reactivity it was less effective in heavily polluted water. Ridenour & Ingols (59) found chlorine dioxide slightly more effective than chlorine against the polio virus.

Because "free" chlorine is such a powerful bactericidal agent Green & Stumpf (67) suggested that it served as another illustration of the "enzyme-trace substance theory," a theory which states that whenever a substance influences biological reactions in trace amounts it must be part of an enzyme or influence or modify an enzyme reaction. These authors observed that the minimal concentration of chlorine required to give 100 per cent inhibition of glucose oxidation by a bacterial cell suspension always completely sterilized the suspension. The time required for the glucose oxidation enzymes to be inhibited was also identical with the time required for complete killing. This is somewhat surprising since one would presume that their respiration measurements could hardly distinguish 99 per cent from 100 per cent inhibition (i.e., if out of a billion cells per milliliter there were ten million or none still respiring) while their cultural methods must have been a million times more sensitive. Of the enzymes involved in glucose oxidation, triosephosphate dehydrogenase was suspected to be the most sensitive and a purified preparation of this enzyme from rabbit skeletal muscle proved to be five times more sensitive to chlorine than the enzyme in the bacterial cell. The authors stated that spores were inhibited by a mechanism different from that encountered in vegetative cells as spore survival did not depend on ability to oxidize glucose. In those spores that did oxidize glucose the enzyme was inhibited by the same concentration of chlorine that inhibited glucose oxidation in the vegetative cell but this destruction of the enzyme did not kill the spore. Since the survival of the spore is determined by its ability to form a vegetative cell apparently the spore subjected to chlorine retains its ability to produce more of the susceptible enzyme while the vegetative cell killed by chlorine loses its ability for enzyme regeneration. Thus the lethal reaction may well be on the gene controlling the enzyme formation which in the spore is better protected from the action of the chlorine.

Wyss & Strandskov (68) observed that solutions of iodine at equilibrium contained I_2, HOI, and I^- in accordance with the hydrolysis and ionization equilibria and that the iodide ion, like

the chloride ion, was inactive. Based on initial dose, iodine itself was three to six times as active as hypoiodous acid. The disinfectant activity of iodine varied with temperature to a great extent and with concentration to a lesser extent than did the activity of chlorine. The presence of excess iodide tied up the active iodine as potassium tri-iodide which was inactive. The germicidal action was not affected by the presence of ammonia or other nitrogen compounds which are not reducing agents.

Salle (69) observed that it was possible to increase the lethal action of iodine manyfold by adding a 1:3000 dilution of an oxidation-reduction system. An effective system consisted of equimolar quantities of manganous sulfate and ferric sulfate. No explanation of the phenomenon was offered. Anderson & Mallman (70) reported that colloidal iodine penetrated faster and therefore was a better killing agent than either an alcohol or water solution of iodine. The tests were made with bacteria suspended in an aqueous medium. Strickler (71) has patented an iodine preparation which is activated by mixing with chlorophyll and red blood cells and subsequently exposing to ultraviolet light.

Since the germicidal action of iodine was not affected by nitrogenous constituents it was found to function more successfully than chlorine in the emergency treatment of small quantities of drinking water (72). A preparation in a readily soluble, solid state would be useful for such purposes and several types have been prepared. Kleinberg, Novak & Gerber (73) prepared and tested the germicidal action of salts of the labile base IOH which was stabilized by coordination of the iodine with pyridine. Iodine acted as a positive ion and some of the salts showed strong germicidal action. Witte (74) reported a stable, soluble preparation made by grinding iodine to a fine powder with glycine hydrogen iodide.

Herbert *et al.* (75) inhibited the action of a purified zymohexase preparation from rabbit skeletal muscle with very low concentrations of iodine. There was no correlation between the inhibition and the number of tyrosine groups on the enzyme. Therefore, the enzyme was not inhibited by iodination of the tyrosine but presumably by oxidation of the sulfhydryl groups essential for the activity of the enzyme protein.

During the recent war the disinfectant action of bromine was investigated to determine if that halogen would serve as a substitute for chlorine and iodine. Krueger-Martius (76) found a bro-

mine preparation to be a satisfactory skin disinfectant. A number of workers (77, 78, 79) investigated bromine as to its usefulness in the disinfection of water and sewage. From their reports it is evident that the effectiveness of bromine is modified by environmental conditions in a manner no less complex than exists with the other halogens.

Wyss & Stockton (80) tested the germicidal action of bromine for spores and vegetative cells and observed that like the other halogens the hypobromite (OBr^-) and the bromide (Br^-) ions were inactive. Elemental bromine (Br_2) was about ten times as active as hypobromous acid (HOBr). Concentration affected the killing action in a manner analogous to that encountered with the other halogens. The temperature characteristic (measured at pH 7.0) was lower than that reported for iodine but higher than that reported for chlorine. Presumably these variations resulted from changes in the concentration of active components present in the germicidal solutions of the halogens as determined by the effect of temperature on the equilibrium constants and did not necessarily imply that different lethal reactions occurred with the different halogens. The presence of nitrogen compounds affected the stability and activity of bromine solutions but the mechansim was different from that encountered with chlorine solutions.

Since Farkas (81) found bromine to be a good *in vivo* disinfectant he compared the amounts of bromine and iodine required to inactivate toxic enzymes (hemolysin, coagulase, fibrinolysin, and spreading factor) from pathogenic cocci. In most instances bromine was three to five times as active as iodine, although in the case of the coagulase from *Streptococcus pyogenes* iodine was more active.

Phenolic Compounds

Jordan & Jacobs (82, 83, 84) carried out an extensive investigation of the dynamics of the germicidal action of phenol using *E. coli* as the test organism. Careful analysis showed that the rate of killing increases with time to a maximum, and then fell sharply at first and more slowly as the surviving population became very small. They employed plate counts for determining survivors and selected as virtual sterilization time (*v. s. t.*) the time required for 99.999999 per cent killing. The equation $C^n t = K$ was satisfactory for determining the concentration coefficient n for temperatures between 20° and 35°C. where it had a value of about 5.5. Outside

this temperature range the value was not constant. The authors made allowance for a threshold effect by plotting log (*v.s.t.*—ten minutes) against concentration. The resulting sigmoid curves could be fitted by the Pearl-Verhulst logistic equation. One of the constants of the equation could be used as a concentration coefficient as it assumed a constant value for all data on concentration.

Jordan & Jacobs (85, 86) observed that none of the commonly accepted temperature coefficients were satisfactorily constant. The Q_{10} varied with concentration and temperature and although almost all values exceeded 2, the range was 1.4 to 29. The temperature velocity constant, μ, calculated from the Van't Hoff-Arrhenius equation varied from six thousand to sixty thousand, although at 26° to 28°C. most of the values clustered around thirty-six thousand. A new relationship was derived again using the Pearl-Verhulst logistic equation. One of the constants in this formula could be used as a temperature coefficient as it assumed a constant value for all the temperature data.

Dallemagne & Derouaux (87) reviewed the literature on substituted phenols and presented data on the influence of a *tert*-butyl group on germicidal action. The increased activity due to the substitution was greatest in the case of guaiacol and least in the case of phenol with the cresols and salicylic acid being moderately affected.

Cooper (88) observed that the addition of acetone to a water solution of phenol or benzyl alcohol increased the bactericidal power more than it did that of *p*-cresol, thymol, or chlorophenols. Tertiary butyl alcohol also increased the activity but glycol reduced the germicidal power of aqueous solutions of the phenols although it increased the activity of aliphatic disinfectants.

Roberts & Rahn (89) observed that concentrations of phenol which kill *E. coli* in ten minutes irreversibly inactivated the enzymes involved in oxygen uptake or methylene blue reduction when acetate was employed as a substrate. The catalase of the organisms was more resistant. The oxidase was completely inactivated by a concentration of phenol which was less than the amount required for growth inhibition, but on dilution the enzyme was reactivated indicating that actual destruction of the enzyme did not occur with limiting concentrations of the disinfectant.

Fogg & Lodge (90) studied the distribution of phenols between olive oil and an aqueous buffer since this was regarded as a model

for the distribution of the disinfectants between the bacterial cell and the culture medium. The compounds (phenol, resorcinol, *m*-cresol, *m*-nitrophenol, *m*-chlorophenol, and *m*-hydroxybenzoic acid) which had high distribution coefficients had a correspondingly large depressant effect on bacterial metabolism. The antibacterial activity of phenolic compounds was thought to be due chiefly to their ability to precipitate proteins, and changes in structure affected mainly the distribution coefficient and consequently the concentration at the site of action.

Salle & Guest (91) patented a germicidal preparation of chlorophenol or sulfophenol, the activity of which was enhanced by admixing with a metallic reducing agent such as ferrous chloride or manganous sulfate. Polyhalogenated phenol derivatives were incorporated into soap by Traub, Newhall & Fuller (92) and were found to lower significantly the average bacterial population of the skin of hands washed regularly with such soap. The effectiveness of other types of germicides in soap was not studied.

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MICROBIOLOGY OF DRINKING WATER AND SEWAGE

By Jan Smit

Laboratory of Microbiology, Agricultural University College Wageningen, Netherlands

Microbiology of Drinking Water

Research in the field of purification of drinking water in recent years has focused primarily on topics of technology, and important and new viewpoints in the bacteriological line have not come to our notice.

The role of bacteria in drinking water has been looked upon quite differently in the course of time, but general opinion may be said to converge on the viewpoint that numbers of viable bacteria are only important as indicators of a possible change in the purity of the water: for potable water of known hygienic quality it is not the level of the bacterial count that primarily matters but unexpected deviations from the daily mean that ask for our special attention.

In this respect the estimation of *Escherichia coli* counts is universally considered of more importance, but methods differ in different countries. Thus standard *E. coli* broth in England is not the same as in the United States and brands of peptones, dyes, and additional substances recommended are widely different. In England the 44°C. test in glucose broth is advocated, which may be considered as a modified Eijkman test. In its original form Eijkman prescribed the use of a solution of 1 per cent glucose, 1 per cent peptone, and ½ per cent sodium chloride in tapwater and incubation at 46°C. Later the temperature was lowered to 45° C. Eijkman's starting point was the observation that water of good hygienic quality never shows fermentation at this temperature, although it may do so at 37°C. This means that the *E. coli* present have lost their power of fermentation at the extreme temperature and conversely this loss, which can be demonstrated by estimating the most probable numbers at both temperatures, provides an indication as to the innocuousness which the water has acquired by natural purification processes. The greater the difference, the safer the water. The new edition, recently issued, of the *American*

Public Health Association's Standard Methods for the Examination of Water and Sewage brings no important changes in the views and recommendations of preceding editions, except that the choice for the partially confirmed test is restricted to the brilliant green bile medium. It is noted that the American Water Works Association recommends its adoption by the different local authorities. As regards the assaying of the number of *E. coli* in samples of drinking water a few new methods are noticed. Thus Calvert (1) advocates the use of tryptose lauryl sulfate broth, this solution giving 5 per cent more positives than does ordinary standard lactose broth. Hunter *et al.* (2) add a confirmatory test in brilliant green lactose bile. Sandholzer & Quimby's procedure (3) is of interest, the lauryl sulfate broth being completed by the addition of sodium nitrate. The sulfate inhibits gram positives, and the development of *E. coli*, which reduces nitrate to nitrite, provides an early presumptive test. Another recommendation for the lauryl sulfate tryptose broth comes from Shane (4), who compared it with brilliant green bile (BGB) broth. During two years 36 per cent of the positive BGB tests were not confirmed. Also when compared with standard lactose broth the lauryl sulfate liquid is regarded as advantageous. Chapman (5) gives a new formula for detecting *E. coli* in water, tergitol-7 and bromthymol blue being added to lactose-proteose agar. Hirsch (6) describes what is called a coliometer, used to estimate the number of *E. coli*. Gas formation is followed by spreading on plates in a closed apparatus, giving quick and reliable results. Grill (7) developed a new method of estimating the number of *E. coli*. A known quantity of water is filtered through a bacteria-tight Zsigmondy-Bachmann filter and the filter layer is then placed on an Endo plate where the colibacteria present develop red colonies. Instead of an agar plate a disk of paper may be used, soaked with a liquid of the same composition as the Endo plate, but without the agar. One might ask whether in all cases the bacteria will lie at such a distance from each other that separate colonies will develop.

The question as to the significance of *E. coli* in drinking water is, however, not solved by this and other means of making coli estimation an easy routine method. The difficulty of this evaluation is once more stressed by an interesting investigation of Schnellen (8), who studied the influence which adsorption of bacterial foodstuffs from very dilute solutions by the sand grains of a slow bac-

teria filter has on the growth of bacteria. Earlier studies of ZoBell (9, 10) and Heukelekian (11) are cited to elucidate the fact that growth and decay of green algae in the filter may so far concentrate foodstuffs on the sand, that development of *E. coli* in the upper layers of the filters ensues. If the deeper layers are clean *E. coli* may pass into the filtered water, but if the deep layers are sufficiently coated the bacteria are held back. Schnellen proved that a decoction of these algae form an excellent medium for growth of *E. coli* and that the concentration of this foodstuff in the filters is too small to allow development unless the absorbing capacity of the sand is able to increase it to the necessary figure. This absorbing capacity may also be the reason why so many bacteria are held back by the upper layers (15 to 20 cm.). In a ripe filter the newly developed bacteria are held back by the sand below, which is not the case in newly filled filters where the deeper layers do not hinder the passing of the bacteria grown in the upper ones. In that case the bacterial count in the effluent may be much higher than in the raw water.

Streptococci in river water form the subject of an interesting study by Hannay & Norton (12). More or less parallel to Dibble's results (1921) on the types of streptococci in feces they found most (60 per cent) of the streptococci in the water of the river Avon to belong to Dibble's group A (he found 40 per cent). Neither streptococci of the type *S. lactis*, nor the gelatin liquefying strains (of which Dibble found nine per cent), were detected. The reviewer is of the opinion that the search for streptococci in drinking or swimming pool water deserves more attention than is often paid to it, especially since their death rate in pure water is high and, therefore, their presence arouses serious suspicion of recent pollution.

A good deal of attention is given to the possible spread of viruses, especially poliomyelitis virus, by water. That polluted creek water may contain the virus of poliomyelitis was proved by Toomey *et al.* (13). They inoculated cottonrats with samples of the water and noted that symptoms of the disease could be induced. The fate of this virus in treated sewage, the subject of many articles, will be treated later. The occurrence of the virus of hepatitis in water was studied by Neefe *et al.* (14, 15). The virus was the cause of a hepatitis outbreak in a camp where 575 cases occurred, following the drinking of contaminated water. It was proved that coagulation and chlorination did not remove nor inactivate it, and

that superchlorination was needed for its inactivation. On the contrary Ridenour & Ingols (16) claim to have proven that the virus is inactivated by free chlorine. The experiments of Leusen, Rhian & Stibbius (17), showed that chloramine is inactive.

An outbreak of gastroenteritis, mentioned by Louis (18), was caused by a cross connection which admitted contaminated water from a creek to the drinking water lines in a factory. After flushing the lines the outbreak stopped. This once again focusses the attention on the potential danger to health of cross connections and faulty plumbing (more common in America than in Europe it seems), notwithstanding all precautions taken and many an article written about this matter in technical papers. An outbreak of typhoid fever in Washington Heights, New York is mentioned in the *Journal of the American Water Works Association*, p. 8, October (1946). Wickersham (19) cites fifty-four outbreaks of typhoid, dysentery, and associated disorders in the United States and Canada between 1920–1936 affecting eleven thousand persons. Forty-nine of these outbreaks are ascribed to faults in the plumbing in hotels, factories, and the like, often unknown to the employers. Shannon & Wallace (20) have made an investigation on the types of bacteria present in dead ends of water mains in Detroit. Among the 448 strains isolated 32.5 per cent were members of the colon group, 20.5 per cent *Alcaligenes*, 14 per cent *Eberthella* and *Salmonella*, 12.7 per cent *Pseudomonas*, and 13.2 per cent *Proteus*.

A survey of the symposium on water bacteriology held at Reading in July, 1947, is given C. B. Taylor (21). E. Windle Taylor (22) mentioned the possibility of infection of water mains brought about by the jute yarn used for joining them. All for the bacteria found were forms of *E. coli*. A similar observation was made by Ferramola (23), who mentions that pathogens also multiply in this yarn. As substitutes rubber or even paper proved satisfactory. Bashford (24) stressed the need for disinfection of cooling water in the canning food industry. Leakage of cans leads to spoilage of their contents from infected cooling water.

C. B. Taylor (25) gives some aspects of the bacteriology of lakes and streams and stresses the need for examination of the bacteria living on plankton and deposits as well as those in the water. An anonymous article in *Water Works Engineering* (26) gives particulars on iron bacteria in ground water and stagnant

lakes containing much carbon dioxide and iron and little oxygen. *Chrenothrix* is the most common organism there, oxidizing organic material to carbon dioxide and oxidizing the iron to the ferric state. The faulty concept is advanced that the oxidation of ammonia furnishes the energy for these organisms. It is, however, the oxidation of iron that is the only source of energy, these organisms not being able to oxidize ammonia.

The need for and the results of disinfection of drinking water is once more stressed by Scott (27), who mentions that measures taken in Connecticut where 95 per cent of the population drinks purified water have decreased the death rate from typhoid from 30.7 per one hundred thousand in 1900 to 0.2 in 1947. Similar figures are given by Muegge (28) for death rates from diarrhea and enteritis of children under two years of age in Milwaukee, these rates diminishing from fifty to a few per one hundred thousand. Disinfection of water for the army by triglycine hydroperiodide is advocated in an article in *Science News Letter* (29). Cysts of amoeba are also killed by this chemical (made available in tablets) from which iodine is split off when dissolved in water. Disinfection of river water in Assam with the aid of silver is mentioned to have been a success (30). Amsbary (31) mentions the usefulness of copper sulfate in combating growths in water mains which hampered the effect of chlorination. Even breakpoint chlorination was ineffective, but copper addition had a preventive effect.

A peculiar method of purifying polluted river water, consisting of artificial aeration by blowing in air, is described by Tyler (32). The success which he mentions will without doubt be a limited one. Breakpoint chlorination is considered a better means of disinfection than is chloramine treatment in an anonymous article in *American Journal Public Health*, Vol. 37 (33). Free residual chlorine eliminated all lactose fermenters, so that the absence of positive presumptive tests is sufficient in cases where no secondary pollution can occur. How strong the need for effective treatment is ensues from the fact, mentioned in this article, that the majority of rural water supplies contained coliform organisms. The ineffectiveness of chloramine, especially at higher pH and lower temperatures, is again stressed by Butterfield *et al.* (34). Some strains of typhoid and dysentery are even less sensitive than are coliforms, except *Aerobacter aerogenes*. They estimate the effec-

tiveness at about 1:25 of free chlorine. With the tolidine-arsenite test the amount of free chlorine may be estimated and so the method of superchlorination may be deemed advantageous for disinfecting drinking water, especially so as this method also solves the taste and odor problem (35). Some specimens of water are not suited to this manner of treatment. Theoretical considerations are given by Green & Stumpf (36), who postulate that every substance which in trace amounts has a profound influence on biological processes must either be an essential part of some enzyme system or inhibit or modify some enzyme reaction. They suggest chlorine inhibits a key enzymatic process, viz., the enzymatic oxidation of glucose, this effect being exactly parallel to the effect of chlorine on the growth of organisms. The time sufficient to stop glucose oxidation was just sufficient to sterilize suspensions of bacteria. The enzyme action inhibited by chlorine was that of triosephosphoric acid dehydrogenase. Oxidation of glucose stops as soon as this enzyme is prevented from functioning. These facts were confirmed with different concentrations of bacteria and various chlorine compounds. Spore bearing bacteria also lose their property of oxidizing glucose after doses of chlorine which do not kill the spores. Monoiodoacetic acid and organomercurials behaved in exactly the same way as chlorine but required more time and the action was reversible, whereas that of chlorine was irreversible. Iodine is weaker and attacks the zymohexase which splits the phosphorylated sugar into three-carbon units. As a cysticidal agent, however, iodine is better than chlorine. These theoretical considerations may prove to be of outstanding value for the promotion of our knowledge in this field. An interesting application has been made by the same authors (37) in the estimation of free chlorine. They studied its influence on the clotting of milk by papain and from the percentage inhibitions noted developed standard curves allowing the estimation of the amount of chlorine. Oxidation without chlorine but by bacteria is mentioned by Hill (38). Loss of ammonia was followed by the appearance of nitrite and nitrate and a decrease of alkalinity and pH, of which facts, according to the author, "a simple explanation could not be found." He thereby overlooks the fact that change of the positive NH_4 ion into the negative NO_2 and NO_3 ions entails a parallel increase in H ions, so that the drop of alkalinity and pH is self explanatory. Bacteria in the floc, formed in this water, are mentioned as is the activity of this floc in oxi-

dizing ammonia to nitrite; but these facts are not recognized by the author as the simple and well-known action of nitrifying bacteria.

The use of chlorine dioxide as a sterilizing agent is advocated in many papers. Generally (39, 40, 41) its effectiveness is deemed as great and under certain circumstances even greater than that of chlorine, and it has the distinct advantage of not imparting any additional taste or odor when employed in a slight excess. This may be caused by the fact (41) that trichlorphenol, which has a very less pronounced taste as compared with *o*-chlorphenol, is formed from phenol. The relative amounts of chlorine water and sodium chlorite solution, from which the chemical is made, may be changed at will, according to circumstances, thus combining the action of free chlorine and chlorine dioxide. The general opinion is that chlorine dioxide is a valuable tool in many water treatment problems.

Corrosion of pipe lines is the subject of several papers. Since the fundamental facts of anaerobic corrosion as a sequel of sulfate reduction were given by von Wolzogen Kühr (42) and those facts were corroborated by Starkey (43, 44) and Butlin & Adams (45) our knowledge of the phenomenon has entered a new field. Parker (46) isolated and studied a new bacterium, *Thiobacillus concretivorus*, capable of oxidizing hydrogen sulfide and corroding concrete by the sulfuric acid formed therefrom. Sulfur is also oxidized to sulfuric acid and this oxidation is accompanied by a drop of pH to less than 2. Pomeroy (47) mentions analogous facts in rotting sewage. The corrosion may be checked by a coating of bitumen. Caldwell & Ackerman (48) stress the possibility of anaerobic corrosion of steel pipes in nitrate containing water. Reduction of this nitrate may indeed cause corrosion, but it is not quite clear from these experiments whether nitrate is essential for this phenomenon, the water in question containing an ample amount of carbon dioxide to explain the corrosion. That corrosion stops when the pH is increased to approximately 8 is not wondered at, and the fact that nitrate reduction is not completely hampered by this increase adds to the probability that carbon dioxide is the major cause. The use of lime to prevent corrosion is advocated by Towbridge (49), who adds this chemical after coagulation and settling. If phenolphtalein alkalinity does not pass through the filter and the pH keeps between 7.5 and 7.6, coating of the sand

by calcium carbonate is prevented and loss of head does not occur. A peculiar fact, worth mentioning, was found by Williams (50). He stated that zeolith softening filters in Louisville, Kentucky, after sixteen days' use with Ohio River water, showed a considerable growth of bacteria and an increasing bacteria and coli count in the filtered water, notwithstanding the fact that chlorine had been applied before filtration. The salient point was the increase in numbers of *A. aerogenes*, compared with *E. coli*, as was shown by cultivation on Endo plates. It is clear from these observations that zeolith filtration calls for an efficient removal of organic substances from the water.

Since excessive growth of algae causes much trouble wherever water is exposed to sunlight, it is not astonishing that the prevention or control of their growth is a topic of much interest. The solution given by Caird (51), consisting in growing fish in the ponds and thereby establishing a natural biological balance, seems reasonable enough, but it is not to be used where purified water is involved. Chlorinated hydrocarbons are advocated by Goudey (52), but they must be handled with care because of taste troubles. The use of chlorine and copper, as is done in most cases, seems to be ineffective (53) when the water is soiled with sewage; but Goudey (54) stresses the point that the effectiveness of chlorine-copper is also influenced by physical and chemical factors in the water. Increase in alkalinity requires more copper, but for every degree of temperature above 15°C. the dosage can be reduced by 2.5 per cent. Amsbary (55) reports the remarkable fact that complete removal of iron is followed by nitrite and nitrate formation from the ammonia present and by growth of *Azotobacter*.

Microbiology of Sewage and Sewage Treatment

In recent years no new developments of importance in the treatment of sewage have been reported. Methods of treatment have been stabilized, and managers of sewage purification plants are on the whole capable of coping with most of the difficulties which different kinds of sewage are apt to cause. Nevertheless, numerous problems in this field remain to be solved and many discussions in print and at meetings of sewage plant managers have taken place.

One of them is determining the types of macro- and microorganisms in different parts of sewage plants and their functions in the process of purification. Howard (56) has given a detailed de-

scription of bacteria and protozoa concerned, especially in activated sludge, whereas Lloyd (57) describes animal life in the different purification processes. Also the interrelation of the various groups of animals is mentioned. Tomlinson's description (58, 59) pertains to animal life in sewage filters in particular. His article is well illustrated. Reynoldson (60) has made observations at the double filtration plant at Huddersfield from which interesting facts may be taken about the development of the film on filterbeds.

That *Psychoda* may be the cause of bronchial asthma is shown by Orman (61). The question of transmission of diseases by sewage is reviewed by Clark (62), and Wilson (63) gives a survey of the many risks of transmission of disease during treatment of sewage. It is proved that many pathogens and eggs and larvae of parasites survive in sewage and sludge but do not multiply. It is true that a progressive decrease of all forms of bacteria takes place with increasing degree of purification of the sewage, but a concentration of all forms of life occurs in the sludge. Stokes *et al.* (64) have found that *Salmonella paratyphosum B* and *S. typhi murium* survived during one hundred eighty days in sludge drying on sand beds and up to forty-five days in digesting fresh solids mixed with ripe sludge. As early as 1942 Kraus (65) studied the spread of tubercle bacilli from the sewage of a sanatorium and found that both the effluent and the slime from a filter contained many tubercle bacilli, originating from water-closets and from poorly disinfected sputa. It is to be emphasized that all products of completely or partly purified sewage are potentially dangerous for the spread of infectious agents and that they are to be handled accordingly. With regard to the virus of poliomyelitis the same may be said, according to an investigation of Gear & Wilson (66), who found the virus in fresh sewage solids, in effluent of humus tanks but not in digested sludge, and in the final effluent that had passed a slow sandfilter. The virus was also present when no cases of the disease were found in the neighborhood. *Psychoda* and birds' droppings were free from the virus. The writers rightly observe that effluent of humus tanks may be deemed dangerous for sprinkling on garden crops. Evans & Osterud (67) made an investigation on the growth of this virus in certain protozoa of sewage. Mixtures of surface waters or sewage, containing protozoa, with the virus failed to show any development of the latter. Cultures of *Bodo, Monas, Oikomonas* and others, grown on wheat extract, mixed with virus, and transferred into new solutions, also gave negative results

when inoculated into monkeys. The protozoa investigated thus prove to offer no nourishment to the virus.

Pillai and co-worker (68) have studied the role of protozoa in the purification process. They cultivated *Epistylis* until free from bacteria and introduced it into a sterile medium. When aerated this culture was as active in purifying sewage as was activated sludge. Their conclusion that the protozoa play the essential part in aerobic purification and that bacteria are only of secondary importance will not be corroborated, it seems, by other workers in the field. Watson (69) studied the mechanism of bacterial flocculation caused by protozoa and found that certain ciliates produce a mucus to which bacteria adhere before being digested. The mucus remains in the neighborhood of the ciliate, and the increase in the viscosity of the medium causes the entanglement and flocculation of the bacteria. Hood (70) also studied biological floc formation and states that it differs from chemical clarification only in means. Aeration involves oxidation, catalyzing activities of oxidizing bacteria. Sewage and natural waters provide the foodstuffs and so the character of the growth depends on their composition. Much stress is laid on the alkalinity and pH of the sewage as influencing the coagulation of the sewage by aluminium sulfate. In chemical coagulation three general zones may be established:

	pH	Alkalinity (p.p.m.)	Result
1.	7.0	100–125	Easy clarification with moderate doses.
2.	7.5	125–175	Less easily clarified with higher doses.
3.	7.5–8	200 and over	Difficult to clarify. Excessive doses.

In biological coagulation more or less the same views hold and here also alkalinity is of major importance, as is shown by studying the buffer values of different sewages. Best results are obtained at low pH-alkalinity values, irrespective of biochemical oxygen demand (B.O.D.).

Sewage oxidation in ponds is studied by Caldwell (71), who showed that shallow artificial basins offering a nominal detention period of from three to four weeks and providing a maximum of photosynthetic activity may show an excellent purification and even a decrease of ammonia, coinciding with an increase of nitrite

and nitrate. The natural purification characteristics of a shallow turbulent stream, as studied by Kittrell & Kochtitzky (72) in the Tennessee River Valley, stresses Caldwell's conclusions in that satisfaction of oxygen demand sometimes proved to be so rapid that it falls out of line with any previous concepts.

Ruchhoft *et al.* (73) add a new and interesting study on the utilization of different organic substrates by activated sludge to the many papers from the Cincinnati laboratories. It was already known that after mixing and aerating sludge with solutions of organic materials part of the latter disappears without any digestion compound being formed. The same holds for different sugars, and the authors prove that these substances are absorbed on the sludge in the first few moments and are partly oxidized afterwards, partly synthesized to sludge material. This absorption is especially quick for dextrin and starch, and repeated feedings of glucose and other sugars increase the removing capacity of the sludge. This rapid absorption of carbohydrates, upsetting the equilibrium of the sound sludge, results in an abnormal increase in sludge of very low ash content, in bulking, and in abnormal growth of *Sphaerotilus*, a well-known phenomenon. Alcohols, amino acids, except tyrosine and cystine, and organic acids are also oxidized and synthesized at an increased rate. No doubt this study will induce several other investigations on the purification by activated sludge. Bulking itself and its causes are described in detail by Enslow (74), who distinguishes peptization of the sludge from swelling of the same. Both phenomena are accompanied by deficiency of oxygen reserve, and increase of the latter may be a corrective, as is proper chlorination. Kraus (75) advocates the addition to the return sludge of digested sludge as a means of combating bulking, which seems reasonable, because it increases the ash content, a low value being one of the characteristics of the disease. The long-known trouble of the rising of activated sludge in the final settling tanks is again mentioned by Sawyer & Bradney (76). It is generally understood that the presence of nitrite or nitrate is essential and that addition of these substances shortens the rising time, as does increase of temperature, but the presence of nitrate does not always involve rising and here the lack of understanding begins. Let us not forget that denitrification, resulting in free nitrogen, asks for two additional circumstances: absence of oxygen and presence of soluble organic material. When purification is pushed so far that organic substances are quantatively oxidized, nitrogen de-

velopment and rising can only occur when, by an excessive detention time of the sludge in the settling tank, new soluble organic material is formed by its decomposition and the amount of oxygen is strongly reduced accordingly. An efficient and continuous removal of the settled sludge, leaving no time for the above processes to develop, prevents rising, notwithstanding the presence of nitrates, as experience has convincingly shown. There are other inhibitors, like chlorine, which kill the bacteria, but thereby the activity of the sludge is impaired. It goes without saying that rising is also prevented when the purification is so far reduced that nitrites and nitrates are practically absent. In this case, however, an early deterioration of the sludge occurs and the extent of purification is soon decreased when the return sludge is not efficiently aerated before being reused.

The estimation of B.O.D. in actively nitrifying sewage is treated by Hurwitz *et al.* (77). They claim that B.O.D. estimates tend to be too high because oxygen is taken up during nitrification. They recommend that this process be eliminated by pasteurization or acidification. Both methods give the same figure, that of the oxygen uptake by carbonaceous materials, without nitrification. In streams the same trend may be seen: when nitrification sets in, B.O.D. increases giving a false picture of purification and stability. Upon acidification the figure found for B.O.D. of the stream decreases gradually. The difference in B.O.D. between the conventional method and modified methods gives the magnitude of nitrification.

The nitrifying capacity of the film grown on a biofilter and a standard filter is studied by Heukelekian (78). During one year the nitrite and nitrate content of the effluent was measured, and at the same time mixtures of film and sewage were aerated during twenty-four hours and nitrification was observed. It was found that the biofilter in this respect was less effective than the standard filter, but that its capacity increases in summer when the amount of sewage applied is less. The impression is gained that the heavy loading of the biofilter is the cause of the low figures found, and that nitrification is not stopped by the large amount of organic material. In both cases the oxidation of ammonia is nearly complete in the upper two feet of the filters. Reduction of total bacteria or of *E. coli* was slightly superior in the standard filter, though in winter this reduction was slight and an increase in num-

bers in the effluent above those in the influent was sometimes found. The uniformity of the effluent of the standard filter throughout the year seemed to be somewhat greater. In this respect a study by Anderson (79) of the toxicity threshold of various sodium salts against *Daphnia magna* is of interest. Among the most toxic are the following sodium salts: arsenite, perborate, chromate, dichromate, cyanide, iodide, sulfide, and thiocyanate. It is clear that these toxic substances must influence the working of sewage filters. The same holds for the pooling of the filters by sulfur bacteria. Shepherd (80) describes a case of heavy pooling by *Beggiatoa alba*. Killing the organism and flooding and flushing did not work, but anaerobic digestion and then flooding was successful. Leakage of ground water of high sulfur content proved to be the source of the growth. Heukelekian & Lassen (81) point to the fact that oxidation of reduced sulfur compounds in sewage is mostly chemical in nature, the rate being the same in sterile and nonsterile sewage. But addition of small amounts of activated sludge enhances this rate. Thiosulfates were the only compound requiring bacteria for oxidation.

Van Kleeck (82) opens a discussion on the merits of different methods of sludge digestion. He begins by pointing out the merits of septic tanks. In former days they were mostly built too small and were rarely cleaned, with the result that the effluent was inferior. But if properly constructed, biological processes may be so active and the effluent may be such that secondary treatment on contact beds or sand filters is quite satisfactory, the over-all removal of solids and B.O.D. being equal and often superior to all other types of sewage treatment. So septic tanks permit an economical design for limited populations at substantial savings in construction and maintenance.

Schlenz & Buswell (83) give general considerations on sludge digestion. Whereas the former author treats the practical aspects, the latter postulates a theory of anaerobic treatment. Most important is the removal of carbon and hydrogen compounds, 75 per cent of which may be digested. Methane production receives detailed attention, and the possibility of starting and stimulating this process by addition of large quantities of cultures of methane bacteria is discussed. Since overloading results in acid formation, lime is often added to avoid this, but according to the author this practice should be avoided as neutralization tends to promote the

formation of volatile acids. Common practice, however, does not corroborate this statement, although it is to be conceded that this neutralization is not sufficient to overcome the trouble of foaming but has to be followed by a better controlled digestion routine. It should not be forgotten that the proper development of the methane bacteria (of which Dr. Buswell gives a good description and beautiful pictures) only takes place in nearly neutral surroundings, so that neutralization is the first step to be taken. The contention of the writers that methane bacteria under favorable conditions may produce acetic, propionic, or butyric acids is not true. Whether the acid producing bacteria, *E. coli* and allied species, will develop depends upon the conditions prevailing in the tank contents as governed by the character of the sludge and the loading and unloading routine. In newly filled and unseeded tanks coliform bacteria generally develop and cause foaming. Once started and fed by frequent strong reloadings, they predominate and there will be no development of methane bacteria unless reloading is stopped and the acid mixture is neutralized. But it is to be understood that the excessive loading and not the neutralization is the cause of the trouble. Seeding with ripe sludge will then be a great help in establishing normal methane fermentation, but it is not strictly necessary as the calcium salts of the fatty acids provide ample nourishment to these bacteria while the acids themselves do not. If reloading is then kept at reasonable figures there will be no more acid formation and no foaming, and methane fermentation will continue undisturbed. Volatile acid control will be a great help, but Schlenz's contention [(83), p. 22] that the calcium salts are unsuited to promote methane fermentation is erroneous.

Keefer (84) investigated sludge digestion at various concentrations of solids. He proved that a 75 per cent moisture content in a sludge treated with ferric chloride is optimal and that reduction to 67 per cent materially retards the digestion. Without the use of ferric chloride digestion in a wet sludge of 95 per cent moisture is most complete. Rudolfs (85) a few years ago studied biological digestion of soluble organic wastes from different industries. Often crude mixtures of fermenting organisms may be used but in other cases pure cultures are to be preferred, and Rudolfs describes a case where the effluent of a yeast factory is treated in three stages, resulting in a reduction of 77.5 per cent. When sew-

age rich in carbohydrates is to be handled it may be advantageous to add nitrogen or phosphorus compounds.

Use is made at an increasing rate of digester gas as fuel for motor cars. Wilson (86) mentions its use for the production of formalin. To this end the gas, mixed with steam, is conducted at 950°C. over a nickel catalyst so that carbon monoxide and hydrogen are formed. This mixture, at a pressure of 2500 lb. per sq. in., is brought in contact with chromite of zinc, where methanol is formed and on dehydrogenation gives formalin. An experimental plant exists at the Johannesburg Public Health Department. Industrial alcohol production from sulfite waste liquor is another example of the use of waste products. Joseph (87) describes the method of the Ontario Paper Company Ltd., where the yeast is recovered by centrifugation and is reused. Methyl alcohol and fusel oil are by-products, and a 43 per cent reduction of B.O.D. is reached. Tyler (88) treats the same subject. For aerobic yeast production *Torula utilis* at 30°C. and pH 4.5 is used and this gives a B.O.D. reduction of 50 to 60 per cent. For alcohol production under anaerobic conditions *Saccharomyces cerevisiae* is preferred, giving a removal of 37 to 46 per cent. This may be in accordance with standards set by State Agencies and would permit the discharge of the treated liquor into streams of sufficient water capacity.

The microbiology of beet sugar manufacture is treated by Allen *et al.* (89). In the return water circuit, where aeration is given and a temperature of 45 to 55°C. is kept, acid is formed by thermophilic lactobacilli and severe corrosion is caused. An amount of 19 p.p.m. of chlorine was needed to keep pH between 5.5 and 6.25 and check corrosion. In the diffusion batteries, where anaerobic conditions and a temperature of 50 to 75°C. prevail, lactobacilli and thermophilic clostridia cause heavy sugar losses and formation of organic acids and gas. Here 60 p.p.m. or more chlorine is needed.

The heavy burden to which streams in the United States are subjected follows from figures given by Wolman (90), to the effect that the raw sewage of forty-seven millions of persons empties in American bodies of water accompanied by untreated and uncontrolled industrial wastes equivalent to fifty-five to sixty million persons. According to Streeter (91) the Ohio River Survey is of the opinion that a most probable number for coliforms of twenty

thousand is just tolerable for a water supply, but that five thousand is more desirable. That algal growth is stimulated by sewage effluents is mentioned by Hall (92) for the Back River at Baltimore which receives sewage with high nitrogen and carbon dioxide content from the Back River Sewage Works. Treatment with chlorine and copper was useless in this case. McLean & Speas (93) have tried to use fluorescence of water as an evidence of sewage pollution. They found that high coli counts are usually accompanied by fluorescence in ultraviolet light. But deep and unsoiled ground water often shows this phenomenon as well, so that the method seems only to be suitable for large plants where daily control is possible, as any increase in fluorescence gives an indication of an undesirable pollution.

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MICROBIOLOGY OF SOIL

By Nathan R. Smith
Plant Industry Station, Beltsville, Maryland

The discovery that certain microorganisms isolated from soil could produce antibiotics when grown in pure cultures in the laboratory has served to focus attention as never before on the subject of soil microbiology. The main emphasis, however, is on problems entirely unrelated to the processes which go on in the soil. The fact that an organism was isolated from soil and then used in medicine, fermentations, or some such way, does not bring such work in the actual field of soil microbiology. Practically all except the more fastidious animal and plant pathogens can be found in the soil at one time or another. Even some of the fastidious organisms are quite regularly found. That is not surprising considering that all sorts of materials find their way back to the soil—the dumping place of much of our refuse. In addition, the soil serves as a good matrix for the preservation of microorganisms, provided competition between the various forms is not too severe.

Waksman (1) reviewed the literature up to 1932, and then supplemented his book with a review (2) covering the years 1932–36. A decade later, Norman (3) very briefly surveyed the field and discussed the status of the science, especially the lack of support allotted to it. It is the purpose of the present paper to review some of the more important work published since 1936. The coverage of the literature cannot be complete owing to the limitation of space; more attention will be paid to some work, less to others. Responsibility for the selection of the references discussed will be the reviewer's, who will be influenced a great deal by his own interests.

Autotrophic Soil Bacteria

Probably the most interesting members of the autotrophic group are those bacteria that oxidize ammonia to nitrite and those that oxidize nitrate to nitrite. They depend upon the carbon dioxide of the soil as the sole source of carbon. Having such a limited physiology naturally makes them rather difficult to handle in experimental work. The presence of some types of organic matter is inhibitive to cultures in the laboratory but does not seem to be

so under natural conditions. Pandalai (4, 5) expressed the opinion that the heterotrophic flora mixed with the autotrophic allowed the latter to function in the presence of inhibitive substances and that this association was symbiotic. The latter view was also shared by Desai & Fazal-Ud-Din (6). Stapp (7) observed that bacteria which were usually chromogenic were frequently associated with the nitrifiers and that they stimulated nitrification. Imsenecki (8) isolated a myxobacterium (*Sorangium symbioticum*, n. sp.) from a clear zone on a silica gel plate which he considered to be identical with Winogradsky's "nitrocystis."

The proper pH value of soil for nitrification has long been a subject for discussion. Using ammonia, ammonium sulfate, and urea in six typical Arizona soils, Caster, Martin & Buehrer (9) found that complete nitrification would not occur above pH 7.7 ± 0.1. There was, however, some nitrite formed even above this threshold. Another interesting point is that ammonia at a concentration as high as three hundred parts per million was not toxic to the nitrifying bacteria and any failure of the ammonia to nitrify to nitrate could be attributed to the high alkalinity of the soil. Analytically they could account for practically all of the added ammonia which indicated that losses by volatilization from the soil or by spontaneous decomposition of ammonium nitrite were negligible. This was not corroborated, however, by Jewitt (10) working with Sudan Gezira soil. Nitrification of various materials in his experiments was normal but appreciable ammonia was lost from alkaline soil when it was added as ammonium sulfate. Fraps & Sterges (11) previously had reported that in the nitrification process nitrogen might be lost by the decomposition of nitrite. Fraps & Sterges (12) also found variability in the nitrification of ammonium sulfate in Texas soils, some nitrifying only 60 per cent of it even though calcium carbonate was added. The addition of phosphate increased the nitrification of most of these, the monocalcium phosphate with calcium carbonate being the best. In this connection, Pikorvska (13) showed that bacteria isolated from different soils varied in their nitrifying ability and Verona (14), that small amounts of phosphomolybdic acid were stimulatory.

The effect of light on nitrification still seems to have its supporters. Dhar & Mukerji (15) again stated that nitrification was due to sunlight alone and that denitrification also occurred with a loss of nitrogen gas. Singh & Nair (16) took a less radical view

and reported that light helped the bacteria to oxidize ammonia to nitrite but did not assist in the further oxidation to nitrate, nor in the ammonification of organic matter. Waksman & Madhok (17) found that biological nitrification was the all important process in the formation of nitrates in soil and that photonitrification did not play an important part in normal soil processes. On the other hand, Puri, Rai & Kapur (18) concluded from their work that nitrites were oxidized to nitrates in soil by a purely physico-chemical process, quite independently of microbiological and photochemical agencies. The oxidation depended upon the base exchange capacities of the soils.

A new and direct method of studying nitrification in soil was proposed by Lees & Quastel (19) which they called the "perfusion technique." By a suitable apparatus, the soil was intermittently perfused by an aerated solution of nitrifiable material. The soil was maintained at near the water-logged state, the excess moisture draining off through the soil. The "perfusate" was then mixed and aerated and again made to drain through the soil. The process was continuous and samples were withdrawn from the reservoir for analysis at various times. One especially interesting fact was brought out by their work, i.e., nitrification in soil takes place wholly at soil surfaces where ammonia is combined or absorbed. An increase or diminution of such receptor sites caused an increase or decrease in the rate of nitrification. The rate is, therefore, a function of the base-exchange capacity of the soil. Little or no nitrification took place in the "perfusate" or soil solution. These and other observations seem to make this a very useful method.

The process of sulfur oxidation by *Thiobacillus thiooxidans* has been elucidated by the work of Vogler & Umbreit (20) and Umbreit, Vogel & Vogler (21). There must be direct contacts between the cells and the sulfur particles in order for the sulfur to be dissolved in the fatlike globules which are usually in the ends of the cells. Umbreit & Anderson (22) observed three types of cells under the electron microscope but the bipolar appearance seen by the light microscope was not brought out. Knaysi (23) found the cell protoplasm gram negative, the vacuolar content, gram positive. One new species isolated from coprolite, *Thiobacillus coproliticus*, was described by Lipman & McLees (24).

The anaerobic reduction of sulfates to hydrogen sulfide by *Vibrio desulfuricans* will be included here although the organism

is a heterotroph. Aleshina (25), supposedly working with this species, reported that chitin was decomposed and ammonia liberated. Butlin & Adams (26), however, were able to demonstrate that it was a facultative autotroph. More astounding was the report by Starkey (27) that spores were formed in cultures isolated at 55°C., whereas no spores were observed in those isolated at 30°C. When first purified, the high temperature strain could be changed into the low temperature and vice versa. Later each apparently became stabilized to its own temperature range, since neither strain could be induced by this reviewer to grow at the other temperature range. A sporulating strain with an optimum temperature of 30°C. was found by Iya & Sreenivasaya (28) in soil where elementary sulfur was being deposited. It preferred 6 per cent of sodium chloride, but would grow slowly without any.

Nonsymbiotic Nitrogen-Fixing Bacteria

The literature on *Azotobacter* continues to be voluminous and often contradictory. Of special interest were the publications by Russian workers in which it was shown that increased yields of many crops were obtained by the application of "azotogen," a peaty material carrying *Azotobacter* cells. This was applied to seed or to the plant roots. Allison (29) has reviewed the literature to which the reader is referred. Allison *et al.* (30), employing two soils in extensive greenhouse experiments, found no significant effect on yield or growth of plants by the use of pure cultures of *Azotobacter* or "azotogen." Clark (31) also obtained negative results in his study of the possible effect of the same materials on the rhizosphere flora. *Azotobacter* added to cropped and uncropped soil disappeared more rapidly from the former. In no case could the Russian work be substantiated. Katznelson (32) reported that soils showed marked differences in regard to their ability to support *Azotobacter* even in the presence of molybdenum, lime, and sources of energy. This inability to survive seemed possibly to be due to unsuccessful competition with other soil organisms, presence of toxic substances, or absence of certain nutrients such as phosphorus or potassium. His experiments indicated that it was possible by soil amendments to establish *Azotobacter* and to stimulate its development in soils originally inimical to it.

The distribution of *Azotobacter* in soils has received considerable attention. Chang (33) reported *Azotobacter*, mostly *A. chro-*

ococcum, in practically all soils of Manchoukuo; *A. vinelandii*, however, fixed more nitrogen. Three-fifths of the Chinese soils examined by Gaw (34) also contained *Azotobacter*. Cultivated soils in Arizona according to Martin (35) usually contained the organism but range soils generally were lacking in it. Sushkina (36) failed to find *Azotobacter* in virgin arid soils in Russia, but after irrigation it appeared even under the meadow type of soil covering. Using the plaque method, positive results were obtained by Peterson & Goodding (37) in 96 per cent of the soils of Nebraska. Their presence was not correlated with phosphorus, exchangeable bases, or pH of the soil. This bacterium was found at pH 5.3, but Stockli (38) failed to find it in soils of a pH value less than 6.0, which is usually considered the critical pH. A salt content of 0.5 to 1 per cent was found to be optimum for *Azotobacter* by Werner (39), which he thought might explain its absence from some soils. The soils of Portugal examined by Louriero (40) were rich in *Azotobacter*, especially *A. chroococcum*. The observations of Swaby (41) are interesting, not because only 26 per cent of the soils tested were positive but because only eight soils had more than thirty *Azotobacter* cells per gram. Similarly, Roberts & Olson (42) found no *Azotobacter*, or, at most, only a few cells, although there was nonsymbiotic nitrogen fixation in their experiments. They concluded some other organism might be involved. *Azotobacter agile* was isolated by Soriano (43) from 25 per cent of the water supplies around Madison, Wisconsin, and San Francisco, California, and in 75 per cent of those around Buenos Aires, Argentina.

One new species of *Azotobacter* has been described recently. Starkey & De (44) isolated *A. indicum* from tropical acid soil (pH 4.9 to 5.2). It grew slower but fixed as much nitrogen as *A. chroococcum* and produced a great amount of tough slime. Although it grew from pH 3 to 9, it lived longer under acid conditions. Another bacterium, which does not belong to this genus, will be included here for convenience. *Azomonas insolita*, isolated by Stapp (45) also from tropical soil, produced acid and gas from most carbohydrates, grew under vary acid conditions (pH 3.3), and fixed a moderate amount of nitrogen. More observations on the occurrence and abundance of these bacteria seem desirable.

Under some conditions, *Azotobacter* may be efficient in fixing nitrogen if associated with other microorganisms. Richards (46) grew *Aerobacter aerogenes* with *A. chroococcum*, the former serving

to break down the starch used in the medium. Nitrogen fixation was shown by Jensen (47) to take place in cultures of *Azotobacter* associated with certain cellulose-decomposing bacteria belonging to the genus *Corynebacterium*, but not when *Cytophaga*, *Cellvibrio*, fungi, or actinomyces were the associated organisms. This was followed by a more detailed study by Jensen & Swaby (48) on the quantitative relationship between cellulose decomposition and nitrogen fixation and the nature of the organic breakdown products of cellulose that serve as energy material for *Azotobacter*.

Hervey & Greaves (49) noted that the presence of ciliates in liquid cultures stimulated nitrogen fixation. Killed ciliates had the same effect but their filtrates or ash had no effect. A chance contamination (probably by *Bacillus circulans*) was reported by Lind & Wilson (50) as being beneficial to *A. vinelandii*. The contaminant made iron available from an old preparation of humates; it had no effect in the presence of fresh humates. Another factor in the fixation of nitrogen is the effect of molybdenum which Bortels (51) discovered and which was corroborated by Horner *et al.* (52) and others. One part per million of molybdenum gave a tenfold to thirtyfold increase in the nitrogen fixed; vanadium was less effective. By adding an "auxin" to agar, Armandi (53) obtained more color and growth of *A. chroococcum* with nearly a twofold increase in nitrogen fixation. Jones & Greaves (54), however, refute the claim that this organism requires certain accessory food substances. A large number of materials were tested but none of them were needed for normal growth and metabolism of the organism.

Azotobacter can utilize a wide range of substrates but not all strains of a species are identical in this respect. Six strains isolated by Guittoneau & Chevalier (55) fixed 9 to 11 mg. nitrogen per gm. of phenol consumed. They also found (56) that strains varied in their ability to utilize sodium salicylate. By adding sodium benzoate or benzoic acid to soil at rates of 2.5 or 5 per cent, Reuszer (57) isolated a strain of *Azotobacter* producing a green pigment although it had never been found previously in that soil. One wonders if aseptic conditions were maintained. Katznelson (32), however, reported that 1 per cent of calcium benzoate, ethyl alcohol, and butyl alcohol completely suppressed *A. chroococcum*. It would appear, therefore, that there is a great variation between species, or perhaps between strains of the same species in their tolerance of such substances, and that benzoates should be used with caution.

Fumes of ethyl alcohol and acetone, however, were readily used by *Azotobacter* according to Kholodny *et al.* (58), whereas vapors of methyl alcohol were unfit. Alcoholic yeast extract (0.5 to 7 per cent) was found by Schroeder (59) to increase growth in proportion to the amount of the extract used. Greaves *et al.* (60) added tyrosine, DL-isoleucine, hydroxyproline, and L-histidine to synthetic mannitol base medium and obtained greatly increased fixation of nitrogen by *A. chroococcum.* Casein and albumin acted similarly but gelatin decreased the fixation. On the other hand, Horner & Allison (61) failed to find that L-histidine was utilized; in fact out of thirty-five nitrogenous compounds tested, including amino acids, purines, pyrimidines, amines, and amides, only urea, aspartic acid, asparagine, adenine, and glutamic acid appeared to be definitely assimilated. As in the case of the long-known effect of nitrate, the addition of nitrogen compounds decreased nitrogen fixation in proportion to the amount of the fixed nitrogen utilized, the unavailable compounds having no effect on fixation. Fedorov (62), nevertheless, found that growth and nitrogen fixation by *A. agile* in solution cultures were stimulated by the addition of 0.01 to 0.5 gm. of *o*-dinitrobenzene per 100 ml. of the medium.

Shtern (63) obtained saltants by irridation which had an increased capacity for nitrogen fixation, the maximum occurring in two days. On the other hand, Dooren de Jong (64) failed to find any permament change induced by treatment with x-ray, and Whelden *et al.* (65) noted a marked decrease in nitrogen fixation proportional to the dosages of the irradiation. In the absence of copper, *A. chroococcum* failed to blacken in Mulder's experiments (66), indicating a beneficial effect of copper. On the other hand, Lewis (67) noted that copper caused a long lag phase in the growth of *A. agile* and that iodine reduced the effect. The ability of the latter to remove the former from solution with the formation of the insoluble copper iodide might easily account for the observation.

Flagellation of *Azotobacter* was restudied by Hofer (68) and all species were found to be peritrichous. Using an old stock culture, Lipman & McLees (69) corroborated previous work of others that a rough black pigmented, a white, or a brown mucoid strain could develop from a parent culture. Emphasis was placed on the appearance of the rough black stage without the use of nitrate, benzoate, or other materials. The dissociation was spontaneous, ap-

parently, and no attempt was made to obtain other forms. The gum produced by *Azotobacter* was analyzed by Cooper *et al.* (70). The polysaccharide was about 90 per cent glucose and 3 to 4 per cent uronic acid residues and belonged to the same class as that of the pneumococcus, Types II and III.

The biochemistry of nitrogen fixation was reviewed and discussed by Burk & Burris (71) and by Burris & Wilson (72). As a result of their own studies using isotopic nitrogen and the work of others, Burris & Wilson (73) concluded that the information at hand favored the view that ammonia was the first stable intermediate formed in nitrogen fixation by *Azotobacter*. Wilson & Burris (74) again reviewed the subject and added to the discussion.

The immediate weather conditions were reported by Bortels (75) to influence nitrogen fixation, i.e., the barometric pressure, moisture, temperature, and light. Seasonal differences in the rate of the process as reported by Roberts & Olson (42) should be expected but one would hardly expect that barometric pressure and light would have any effect. The latter suggests the work of Dhar and his co-workers in India, who have published a series of papers over a period of years on the photochemistry of nitrogen fixation in tropical soils. In a recent paper, Dhar (76) still reports extraordinary gains in nitrogen attributed to the effects of light.

One very curious observation was published by Peklo (77). Aphids, beetles, etc., were found to have *Azotobacter* associated with them which enabled them to gain in nitrogen and fat. He apparently crushed the insects and stained the smears, since no mention was made of any cultures. In view of the extensive work of Steinhaus and others (78) it is very doubtful if Peklo's observations can be substantiated.

Nitrogen may be fixed by other microorganisms in the soil, although they have not received as much attention as is given to *Azotobacter*. De (79) reported fixation by blue-green algae in rice fields, but no benefit of growing *Azotobacter* and algae together. Similarly, Stokes (80) reported that no nitrogen was fixed by mixed cultures of *A. chroococcum* and green algae, owing to the small amount of organic matter secreted by the algae. Bortels (81) included *Nostoc* in his studies of the effect of molybdenum on nitrogen fixation. Very little work seems to have been done on *Clostridium*, at least, in its relation to soil processes. Jensen (82, 83) in

laboratory cultures demonstrated that nitrogen was fixed by *C. butyricum* in symbiosis with certain cellulose decomposing organisms and that molybdenum was essential for the process, as has been noted above for other organisms. Nine strains of *C. butyricum* and one of *C. acetobutylicum* were used in the latter work and in case of five strains of the former, vanadium could replace molybdenum. He stated

> It thus appears that molybdenum, partly replaceable by vanadium, is a specific catalyst of nitrogen fixation in *C. butyricum* as well as in *Azotobacter* and probably in other nitrogen-fixing forms of life.

Symbiotic Nitrogen-Fixing Bacteria

The beneficial effect on soil and the succeeding crop of growing a legume has been known for ages and it is equally well known that there exists a symbiosis between the plant and the bacterium which inhabits the nodules on its roots. Because of the great economic importance of legumes and the scientific interest in nitrogen fixation a tremendous amount of research has been carried on, both on the microorganism and on its relation to the plant. But, as yet, the secrets of the association are still unsolved. The literature on the biochemistry of symbiotic nitrogen fixation was adequately reviewed by Wilson (84).

The symbiosis must depend upon a delicate balance of factors, either as concerns the plant or the microorganism. Chen & Thornton (85) favored the idea that the poor growth of ineffective strains of the bacterium in the nodules indicated that tissues of the host plant provide an environment that was less suited to the ineffective than to the effective strains. Whether this unfavorable factor is normally present in the plant or whether it appears as a consequence of infection by the ineffective strain is not clear. They did find that the ineffective nodules began their growth quite normally and only later showed arrested development. In fact, their data showed no evidence that ineffective strains were really less efficient than the effective strains in fixing nitrogen, if the unit of bacterial mass in the nodules and the length of time before the nodules collapse and disintegrate are considered. One wonders, therefore, if the commonly used term of "parasitic strain" really indicates that condition or whether the blame for the lack of complete symbiosis should not sometimes be placed on the plant.

Nutman (86) approached the problem of "effective and ineffec-

tive strains" from the angle of the genetic constitution of the plant. He found that among many thousands of plants raised from commercial seed, one plant was "resistant" to normally effective bacteria. From this plant a "resistant" line was raised. This factor, however, was only one of several which influenced the symbiosis. He drew the general conclusions that the number and activity of nodules formed on the plant depended upon the interaction of factors, in the bacteria and in the plant, both liable to change by mutation. The relationship, then, would seem to be much more complex than is generally supposed.

This complexity was emphasized by the results obtained by Vincent (87, 88) and later by Erdman (89), both working quite independently. They found great variability in the effectiveness of various strains of *Rhizobium trifolii* on *Trifolium*. This indicated the necessity of selecting proper cultures to obtain maximum nitrogen fixation and growth of the plant. It would seem that the work of these two investigators complicates the manufacture and distribution of cultures which would produce maximum benefit to the plant. This point was brought out further by Nutman (90) wherein he reported that stock cultures of the effective and the ineffective variants maintained on agar showed an occasional tendency to produce new variants in effectivity. If the effective strain was stored in sterilized soil, a considerable proportion of the population proved to be ineffective. Reversion to the effective parent type by plant passage occurred in only two out of more than thirteen thousand nodules. Plant passage had no effect upon the ineffective stock culture. Although conditions of the experiments were different, these results would seem to be at variance with those of Krassilnikov (91). After prolonged culture in filtrates of clover, he found that nodule bacteria from vetch, pea, alfalfa, and bean acquired the ability to form nodules on clover. Fermentative changes were also noted, but not cultural or morphological. Whether inoculation by an effective strain can occur after an infection by an ineffective strain was restudied by Virtanen & Linkola (92). It was established that effective strains could not usually form nodules after a prior infection by an ineffective strain. Differences in this respect were noted, however. They ascribe this to an immunity in the plant set up by the first infection. An attempt was made to divide the roots and inoculate the two halves with effective and ineffective strains, but the results

were inconclusive, owing perhaps to the lack of complete bacteriological control.

The question of nitrogen fixation by excised nodules was reexamined by Machata, Burris & Wilson (93). Isotopic nitrogen and postulated intermediate compounds were used in these experiments with inconclusive results. During five years of study on the subject in their laboratory, one hundred and thirty-three samples of nodules from plants grown under a variety of conditions and subject to numerous diverse treatments were studied. It was concluded that unequivocal evidence of nitrogen fixation was lacking and that the inconsistent results reported in the literature may well have arisen from inadequate bacteriological control, most of the nodules having been grown in unsterile conditions in regard to other bacteria.

The fact that molybdenum was essential for nitrogen fixation by *Azotobacter* suggested to Jensen & Betty (94), among others, that it might also be essential for symbiotic nitrogen fixation. Their results indicated that molybdenum stimulates the process, besides presumably being required for general metabolism of the plant, and that vanadium cannot replace it. Anderson (95) reported the astounding fact that one ounce of molybdenum trioxide per acre gave a high order of increased yield of clover in pastures in Australia. This work was extended by Trumble & Ferres (96), and others, and clearly showed the need of this element, especially on the sandy soils in the mountainous regions of South Australia.

Attempts have been made in the past to separate the various species of *Rhizobium* by serological methods. Vincent (97, 98) studied the alfalfa and clover bacteria and found great heterogeniety among strains of each group, no relationship existing between the host species and the serological reaction of the organism. Kleczkowski & Thornton (99) reported similar results. Strains derived from peas showed a close resemblance in agglutination reactions to others derived from clover; on the other hand, strains that were totally unrelated in antigenic structure were found among those isolated from either one of the host plants. In a way, this bears out the contention of Wilson (100, 101) that the cross-inoculation groups should be abandoned because nodulation occurred quite promiscuously in his experiments. Further evidence for this position was given by Wilson & Chin (102) in work on the

root-nodule bacteria associated with species of *Astragalus*. The criterion for this work was the production of nodules without regard to whether the plant was benefited or not. Naturally, this has led to considerable criticism. It should be remembered, however, that, as noted above, effective and ineffective strains may be produced in the same nodule and that the latter may originate from the former in a test tube. We should not, therefore, for purposes of taxonomy, require that nodules should be beneficial to the host plant. This reviewer has believed for a long while that only one species of *Rhizobium* should be recognized and that effective strains should be considered as varieties and carry the name of the host plant. It seems to him that the confusion now existing would be clarified. No one could object to a variety of a *Rhizobium* species changing into another variety, whereas if these same organisms were designated as species, he would have valid grounds for objecting (i.e., one species changing into another species).

The significance of hemoglobin in the nodules first noted by Keilin & Wang (103) was reviewed by Virtanen (104) and further discussed by Keilin & Smith (105). No unanimity of opinion has been reached except that its presence in the nodule is correlated with nitrogen fixation.

After a study of the vetch nodule bacterium in culture, Gaw (106) concluded that the morphological variations observed in no sense represented stages in a life cycle and that regular stages were not passed through. Lilly & Leonian (107) also working with pure cultures demonstrated that there was a close relationship between the iron content of the medium and the need for accessory growth factors. As to the effect of growth factors on nodule formation, Guyot (108) reported that thiamine increased nodules on alfalfa and peas but the results were erratic on beans, peas, and soybeans.

Various explanations have been suggested for the decline of the legume bacteria in soil. In the past the main emphasis has been placed upon the presence of a bacteriophage. Vandecaveye *et al.* (109) presented more proof of the presence of a phage in soil and nodules. The only logical explanation for the poor growth of alfalfa in their experiments seemed to be that the lysis of the alfalfa nodule bacterium caused a drastic reduction in symbiotic nitrogen fixation. Katznelson & Wilson (110) made a survey of soils in New York State and found the phage present in practically all the alfalfa fields examined. They appeared to be undecided whether

this should be considered a normal condition or whether it might be a factor in limiting symbiosis.

The question of whether antibiotics were active against the nodule bacteria was investigated by Trussell & Sarles (111). In liquid culture, certain strains were definitely adversely affected. Taking this as a lead, Robison (112) isolated from soil by the giant colony technique two cultures each of bacteria, actinomyces, and fungi which were antagonistic to the legume bacteria. When these were added to sterilized soil in a greenhouse experiment, they appeared to interfere with nodulation. This would suggest that antagonists may be responsible for the decline of legume bacteria in soil.

Autochthonous Soil Bacteria

A large percentage of the colonies appearing on plates made from a soil suspension belong to a group commonly called the "autochthonous" or the "indigenous flora." They are usually slow growing and not very active physiologically and consequently some are difficult to classify. They have been studied by various workers but their identity and relationships have not been fully worked out. Topping (113, 114) made an attempt to group some of them according to their morphology and reaction to Gram's stain. Both of these characters, however, are quite unstable in this group. She did demonstrate that higher plate counts could be obtained if yeast extract was included in the medium. Lochhead and his co-workers (115, 116) attacked the problem of characterizing this group by determining the nutritional requirements of the organisms as regards amino acids, specific growth factors, yeast extract, and soil extract. Seven groups were established, varying from those bacteria that would develop in the simple basal medium to those that failed to grow even with the above additions unless soil extract was also added. The latter group comprised 19 per cent of the isolates from soil. The factor, or factors, present in the soil extract was not concerned with the ash constituents but was present in the acetone extract and was adsorbed by charcoal and recovered by elution. It was noted that the extract from a fertile soil was much more effective than that from a poor soil. Topping (114), however, failed to find that soil extract had any special value. No doubt variations in methods of preparation have been factors in studies of soil plating media.

More work, especially with divergent soil types, should be done to determine the relative value of soil extracts.

It is doubtful whether the nutritional grouping will be of any value as far as bacterial classification is concerned. It has, apparently, been of value in determining shifts in population due to soil treatment. For instance, Hildebrand & West (117, 118) found that soybeans, carbohydrates, and acetic acid induced an increase in bacteria requiring known amino acids and growth factors and a decrease in those with very simple nutritional needs. Incidentally, this shift in population was associated with a decrease in strawberry root rot in that soil. Katznelson & Chase (119) corroborated the observation of Taylor & Lochhead (120) that in a soil of a given type the relative incidence of the nutritional groups is very similar regardless of the fact that one was a fertile and the other a poor soil. Easily decomposable materials stimulated a temporary change, whereas slowly decomposable substances had a more profound and persistent effect.

The function of the autochthonous flora is probably concerned with the decomposition of the more resistant soil organic matter rather than with easily and quickly decomposable substances which are sometimes added to soil. Certain groups, therefore, should be expected and have been found to be able to attack ring and heterocyclic compounds. Plotho (121) isolated from soil strains of *Proactinomyces* which had this ability, each strain being specific for a particular substance. The mechanism of the oxidative destruction of the benzene ring was studied in more detail by Evans (122), who also included a good review of the literature on the utilization of aromatic compounds by soil microorganisms. A pure culture of a *Vibrio* oxidized completely both phenol and benzoic acid with the formation of intermediate products which were confirmed by isolation. Cholesterol was found by Turfitt (123) to be decomposed by two new species of *Proactinomyces*.

Additional work was done by Taylor & Lochhead (124) and by Taylor (125) on the occurrence and characterization of *Bacterium globiforme*. Of ninety soils selected from various fields in Canada, eighty-nine contained this organism. Large numbers were present if the reaction of the soil was above pH 5.0, but no relationship with soil fertility was found, although higher numbers were sometimes found in fertile soils owing to the greater bacterial population.

From time to time, studies are made on the relationship of the

crown gall organism and its nonpathogenic counterpart found in soil. These species are now included in the new genus *Agrobacterium* in *Bergey's Manual* [(126), p. 227] and, as the classification indicates, they are very close systematically. Coleman & Reid (127) in a serological study of *A. radiobacter* and *A. tumefaciens* found that they were identical in the S phase but dissimilar in the M phase. Their results suggested that the two species represented a single species and that in their M phase they bore to each other the same relationship that had been found between the various types of the pneumococcus. Studies on the nutrition of these two organisms by Starr (128) verified the previously reported fact that these two species could grow in a purified inorganic medium whereas certain other species of the genus required vitamins. The work on the S and M phases had not been published when he did his work and he did not go so far as to suggest that they might be identical except for pathogenicity.

Although not a function alone of the autochthonous flora, the decomposition of hydrocarbons will be mentioned here. ZoBell (129) reviewed the extensive literature on the subject and concluded that hydrocarbon-oxidizing microorganisms were widely distributed in soil, water, and recent marine sediments and especially abundant, of course, in oil-soaked soil. The nature of the organisms varied greatly, nearly a hundred species of bacteria, yeasts, and molds having been shown to have that ability.

Sporeforming Bacteria

An antithesis to the autochthonous flora considered above is the rapid growing versatile group of aerobic sporeformers. They generally constitute only 5 to 10 per cent of the soil flora and probably are not important functionally except in special instances. During the past decade they have been extensively studied as to their characterization, classification, and relationships. The fact that they may appear anywhere because of the formation of heat and drought resistant endospores makes them of general interest to those working in food, dairy, medical, and other laboratories. A special interest has recently been shown in certain species due to their ability to form antibiotic substances. Smith, Gordon & Clark (130) obtained and studied a large number of authentic named species of the genus *Bacillus*, and also included numerous isolations from soil. The variability of each species was determined

so far as possible from laboratory studies, in other cases by the appearance in the collection of variants listed as individual species. Cognizance was taken of the different stages of growth, i.e., rough, smooth, mucoid, rhizoid, and dwarf, and of variation in other characters. Many named species were, therefore, found to be merely stages of growth or variants of a "basic species." For instance, *Bacillus subtilis*, which normally had a rough surface, might appear in bakery products as a slimy organism which had previously been called *B. panis;* if the growth had a folded surface, it was either *B. vulgatus* or *B. mesentericus* (European strain); if a black pigment was formed, it was either *B. niger* or *B. aterrimus;* and if red, it was *B. globigii.* Many of these variants spontaneously changed to the basic species (*B. subtilis*), others were more stable and required considerable manipulation to induce the change. Lysis by a particular bacteriophage isolated from soil was used to good advantage on certain species, especially *B. cereus*, *B. megatherium*, *B. pumilus*, and *B. brevis.* The fermentation of carbohydrates was valuable if ammonia nitrogen instead of peptone was used in the basal medium, and a liberal interpretation was placed on the results. Adaptive enzymes were often found. Many strains unable to utilize a particular carbohydrate could be induced to do so by ageing and serial transfer on the medium containing that substance.

Of special interest was the finding that the rhizoid *Bacillus mycoides* would easily dissociate into a nonmotile *B. cereus* and that these dissociants could not be distinguished from certain cultures of *B. cereus* of soil origin or from nonpathogenic cultures of *B. anthracis.* They postulated, therefore, that *B. anthracis* was a pathogenic variant of the soil *B. cereus.* For convenience, however, *B. anthracis* was retained as a separate species by Smith in the sixth edition of *Bergey's Manual* (126, p. 706).

Gibson (131, 132) and Gibson & Abdel-Malek (133) studied the *Bacillus subtilis* group and came to practically the same conclusions as were published somewhat later by Smith *et al.* (130). The two groups of workers disagreed in one point, however; Gibson maintained that *B. licheniformis* was distinct from *B. subtilis*, whereas Smith and his co-workers considered it as a vigorous strain of *B. subtilis.* Lamanna (134) by the use of precipitogens from spores corroborated Gibson's results but he made different recommendations as to the names to be used for the two species.

Lemoigne and co-workers (135) studied the production of β-hydroxybutyric lipids and acetylmethylcarbinol as a means of separating the genus into four divisions, namely, those species positive in both respects, those negative in both respects, and those positive in one and negative in the other test.

Cellulose Fermentation

The fermentation of cellulose by soil microorganisms still seems to be a fruitful field for research. Fuller & Norman (136) isolated and described five new species capable of fermenting cellulose to a greater or lesser degree; three species of *Pseudomonas*, one *Achromobacter*, and one *Bacillus*. They found that the presence of xylan in corn stalks allowed a greater destruction of the cellulose and that, in the case of vigorous bacteria, the decomposition of cellulose increased as the lignin decreased. With weak organisms, no difference was noted. They thought that the inhibition of lignin was mainly physical. Alarie & Gray (137) isolated from Quebec soils thirteen cultures of aerobic bacteria that decomposed cellulose, eight of which they assigned to new species; five to the genus *Bacillus*, two to *Vibrio*, and one to *Bacterium*. These were briefly characterized and apparently no effort made to compare them with known species, nor was there any quantitative determination made of the cellulose decomposed. This was apparently slight in most cases, filter paper breaking in a peptone solution usually in one to three weeks. With a majority of the cultures, growth on cellulose agar failed to give a clear zone. Two of the new species of the genus *Bacillus* were separated merely on the ability to ferment dulcitol. These and other considerations suggest that a more thorough study of these newly named species is very essential.

Perlin, Michaelis & McFarlane (138) used an impure culture of one of the above new species (*Vibrio perimastix*) and determined the products of decomposition. It was brought out that alkali treated cellulose was more easily attacked than untreated cellulose, 30 per cent of the former and 15 per cent of the latter being decomposed in two weeks.

The fermentation of cellulose by the *Myxobacteriae* has been studied vigorously by many investigators. Stanier (139) summarized the work up to 1942 and included a proposed classification and a brief description of the species. Fuller & Norman (140) extended the information on the *Cytophaga* group and named three new

species, two of which had a weak action on cellulose which was soon lost in the laboratory. The physiology of these isolates was more versatile than noted in other species, which necessitated a revision of Stanier's key to permit the inclusion of those forms. The question of whether *Cytophaga* could use glucose was settled in the positive by Fåhraeus (141) and Stanier (142); the former later reported (143) that cellulose was split to glucose which was consumed at once. He thought that the mucilage found in cultures was synthesized from simple compounds.

The *Myxobacteriae* decomposing chitin were studied by Stanier (144) and found to belong to the genus *Cytophaga*. Their nutrition was unspecialized and good growth occurred on a variety of media. Singh (145) observed that various species produced an extracellular enzyme capable of passing a cellophane membrane, which could lyse gram negative bacteria to a greater extent than the gram positive.

In soils more acid than pH 5.0, Skinner & Mellem (146) found fungi active, whereas if the pH was above 5.0, both fungi and bacteria were responsible for cellulose decomposition. Various other factors were examined by Reese (147) and two methods for studying cellulose decomposition quantitatively were worked out which involved nutrition and aeration. *Sporocytophaga myxococcoides* and *Cellulomonas* spp. were used. These bacteria were found by Jacobs & Marsden (148) to be inhibited by a substance in sawdust from a variety of coniferous trees. The toxic material could be extracted from the sawdust with water or better with a mildly alkaline solution of inorganic salts. The residue, however, was still very toxic and completely inhibited *Sporocytophaga*, whereas the action of *Cellulomonas* was delayed but not prevented. This antibiotic is probably not that isolated by Frykholm (149) from *Pinus silvestris* and named "pinosylvine" by him. But it is apparently identical with the water extract from Western Red Cedar as reported by Southam (150). A wide variety of bacteria and fungi was found to be inhibited but not killed.

The decomposition products of cellulose under anaerobic conditions were acetic and butyric acids as determined by Pochon (151). The anaerobes were unstable in culture and frequently lost their cellulolytic properties. Rotmistrov (152) considered the anaerobic cellulose bacteria as butyric acid organisms and together with Lokhvitskaya (153) isolated from soil several strains

of *Clostridium butyricum* which after five to seven weeks fermented filter paper like true anaerobic cellulose bacteria.

Under thermophilic and anaerobic conditions, Rotmistrov (154) found that *Clostridium illipsosporogenes* n. sp. produced 5 to 10 per cent of alcohol and 45 to 70 per cent volatile acids. Pochon (155) also isolated a new species (*Terminosporus thermocellulotyticus*) which produced acetic and butyric acids and some alcohol. But in studying this organism, Pochon & Sarciron (156) found practically as good decomposition of cellulose under aeration. In this connection, Murray (157) showed that the bacteria usually considered as anaerobic were really aerobic or facultative, and that humidity was the critical factor in aerobic cultures, saturation of the air with moisture being necessary.

The saprophytic chytrids were shown by Whiffen (158) to have some power to dissolve cellulose, varying from a weak to a fair fermentation (35 to 65 per cent decomposed). Stanier (159) demonstrated that the chytrid *Rhizophlyctis rosea* was able to attack cellulose. His results showed also that cellulose or its hydrolyic products, cellobiose and glucose, were its chief, if not its only, carbon source.

Changes in the Soil Population

Effect of herbicides on the soil population.—In recent years the use of herbicides has increased tremendously, especially since the organic forms have been made. Most popular of the latter are various derivatives of 2,4-dichlorophenoxyacetic acid (2,4-D). Smith *et al.* (160) found no significant effect of 2,4-D on the total plate counts, actinomyces, fungi, and protozoa at concentrations up to 500 p.p.m. The nitrifying bacteria, however, were definitely injured with 100 p.p.m. but they recovered in from ten to forty days. The nitrite-forming group was more sensitive than the nitrate-formers. The applications of this herbicide used in these tests were considerably greater than recommended in practice. Payne & Fults (161), however, found that as little as 0.009 lb. per acre drastically reduced the nodulation of beans grown in treated soil and that 0.075 lb. entirely prevented nodulation. In this case, the injury may have been on the plant rather than on the legume bacteria.

The effects of chlorate are more severe. Lees & Quastel (162) noted a bacteriostatic action on the nitrate-forming bacteria which

caused an accumulation of nitrite in the soil. No bad effect on the nitrite-formers was apparent. Smith and his co-workers (160), however, found a great reduction in the numbers of nitrite-formers when 500 lb. per acre of sodium chlorate were added; no determination was made of the nitrate-formers. Although these investigations did not agree exactly, they showed a definite toxicity of chlorate to the nitrifiers. Nelson (163) increased the bacterial activity by adding organic matter to a chlorate treated soil in a laboratory apparatus. Under practically anaerobic conditions, the toxicity of the chlorate was reduced whereas when nitrate was added, the toxicity still remained. Obviously more work on this herbicide is indicated under better controlled conditions.

Ammonium sulfamate, sodium arsenite, and sodium borate were not harmful to any of the soil microorganisms according to Smith *et al.* (160). Ammonium thiocyanate, on the other hand, was inhibitive and bactericidal, but the fungi were stimulated. This probably was due to decomposition products, perhaps hydrocyanic acid.

Effect of insecticides on the soil population.—Highly chlorinated hydrocarbons have recently been developed as insecticides. They are not only used on plants but also in soil, as in the control of the Japanese beetle, wireworms, etc. The effects of dichlorodiphenyltrichloroethane (DDT) were studied by Wilson & Choudri (164) and no injury was noted on ammonification, nitrification, the soil population as determined by the plate counts, and the nodulation of alfalfa, red clover, soybeans, and vetch. Pure cultures of various bacteria, actinomyces, and molds were also not affected. Appleman & Sears (165) likewise did not find any interference with nodulation of legumes when less than 100 lb. of DDT were applied per acre. Heavier applications adversely affected nodulation. Payne & Fults (161) found more injury than this, the number of nodules on bean roots being reduced more than a half by 103 lb. per acre.

Benzene hexachloride (BHC) and chlordane proved to be quite toxic to the nitrifiers, especially to the nitrate-formers, in experiments conducted by Smith & Wenzel (166). A fungicidal action was also noted when a heavy application of 500 lb. per acre was added. In the same tests, a chlorinated camphene had no harmful effects on any of the groups of soil microorganisms. In none of these experiments did the protozoa of the soil appear to be affected.

On the other hand, Lloyd (167) reported toxicity to *Paramecium candatum* when more than 1 p.p.m. of BHC was present.

Effect of organic matter on the soil population.—A great amount of work was done in the early years of the science on the decomposition of organic matter in soil. Much of the research was of a biochemical nature and only casual attention was paid to the organisms involved. In later years analyses made during the decomposition process included a determination of the groups of soil organisms. In a paper by Lockett (168) young and mature rye and clover was said to increase the bacteria, actinomyces, and fungi; the extent of the increase depending upon the nature of the organic material. Stevens (169) added the factor of irrigation to cropped and fallow field soils. In his experiments, the microbiological results correlated well with crop production. Bodily (170) used dried and finely ground green manures in soil and found results similar to those that had previously been reported when the fresh green material was added. The increase in numbers of bacteria reached a peak in three days and then dropped rapidly to the sixth day, after which there was a slow decline.

King (171) found that large amounts of stable manure caused great increases in soil microorganisms and postulated that perhaps this caused the reduction noted in the activity of the cotton root rot fungus, *Phymatotrichum omnivorum*. As a result of this work, Mitchell *et al.* (172) undertook to determine the course of the soil population over a period of a year in the black soils of Texas. Soils cropped to continuous cotton and those receiving sorghum and cowpea residues were analyzed for groups of microorganisms. Plate counts far in excess of those generally reported were obtained, the peak coming in April. In the extension of this work Mitchell, Hooton & Clark (173) and Clark (174) reported that the sclerotia of the fungus could be destroyed in soil devoid of susceptible roots by adding organic matter. Especially important was the observation that cutting below the crown encouraged saprophytic fungi and hastened the disappearance of *Phymatotrichum omnivorum* from diseased cotton root systems. It was concluded from these observations that by exploiting microbial antagonisms a practical line of attack against root-rooting parasites could be undertaken.

Fixation of minor elements by soil microorganisms.—The liming of acid soils has been said to reduce the availability of boron. At first, it was thought to be a chemical fixation, but later it appeared

to be biological. Hanna & Purvis (175) measured the carbon dioxide evolved from an acid and limed soil which showed an increased microbial activity especially in the limed soil. The influence of added boron also was greater in that soil. From plate counts, the fungi seemed to be more affected than the bacteria and the possible use of *Trichoderma* species was suggested as a test for boron deficiency. Previous to this work, Ark (176) attributed the little-leaf or rosette disease of fruit trees to a zinc deficiency. He found that the healthy soil contained mostly fungi whereas the diseased soil contained mostly bacteria. Soil sterilization cured the trouble, as did applications of zinc. He isolated three bacteria, two of which produced the disease when inoculated into healthy soil.

A deficiency of manganese has been said to cause the "grey speck" disease of oats, especially on alkaline soils. Various explanations have been offered for the appearance of the disease but none of the chemical or physical factors seemed to fully explain its cause. MacLachlan (177) isolated manganese-oxidizing bacteria and fungi and attributed the deficiency of available manganese in the soil to microbial activity. Although not connected with any study of this disease, Marsh & Bollen (178) obtained an increase in the mold count on certain Oregon soils, a decrease on one, and an increase in the bacterial count in a peat by adding manganese. Carbon dioxide production indicating microbial activity responded roughly inversely to the available manganese present in the soil. Timonin (179) found that a susceptible variety of oats harbored around its roots a denser population of manganese-oxidizing, casein-hydrolyzing, and denitrifying bacteria than a resistant variety when grown in the same soil under identical conditions. Sterilization of the soil by fumigants reduced or completely eradicated the bacteria capable of oxidizing manganese. A positive correlation was obtained between severity of the disease and manganese-oxidizing and cellulose-decomposing microorganisms.

It would appear, therefore, from these and other publications that soil microorganisms may be instrumental in immobilizing some of the minor elements and thus upsetting the nutrition of the plant.

Microorganisms on the Roots of the Higher Plants

The mycorrhiza.—The literature on the ectotrophic and endotrophic mycorrhizal flora affecting trees has been quite adequately

reviewed by Rayner & Neilson-Jones (180) and by Schmidt (181) and need not be repeated here. The status of the problems was aptly analyzed by the former:

A traditional atmosphere of controversy envelops attempts to unravel the tangled skein of mycorrhizal relationships. The habit is so wide spread among vascular plants, and its expression in different groups so varied that contributions to the elucidation of the problem as a whole are necessarily fragmentary.

The mycorrhiza of crop plants has received less attention than that given to trees. Bain (182) reported that the myorrhizal flora of cranberries consisted of four unidentified fungi. There was no indication that these were necessary nor that they produced any injury. Systemic infection was lacking. Magrou (183) noted that potato roots had more mycorrhiza in good soil than in poor soil, and healthy roots more than unhealthy roots. Previously reported fixation of nitrogen by mycorrhiza was refuted by Bose (184).

The rhizosphere.—The soil immediately surrounding the root has been considered by some investigators as representing the rhizosphere, whereas others have included the roots, or their surfaces, with the adjacent soil. This naturally has led to some confusion since the most abundant flora is on the root surface. If a bacterial analysis of the soil adhering to the roots is made, then the moisture content of the soil from which the roots are taken is an important factor. According to Clark (185), roots from comparatively dry soil gave much higher numbers of microorganisms than roots from a moist soil. He attributed this to the adherence of more soil of a lower microbial content to the moist root. In fact, this was proven by analyzing roots from dry soil and from the same soil to which water had just been added. It is obvious, therefore, that some way of reporting results obtained by the plate count method should be worked out to make the data accumulated by different workers comparable.

The use of the buried slide (Cholodny technique) was suggested by Starkey (186) for studying the flora of the rhizosphere. Results were analogous to those obtained by the plate count method. Linford (187) grew seedlings in a glass chamber made of rings and cover slips and made direct observations on the roots magnified up to nine hundred diameters. He confirmed Starkey's observation that microbial activity was not confined to the older roots but also occurred on root hairs.

Modification of the flora of the roots of wheat by adding organic matter was not successful in Clark's experiments (188), al-

though a great increase in microbial numbers occurred in the soil. On the other hand, Morrow *et al.* (189) observed that organisms inoculated on the seed or seedling could be recovered later from the rhizosphere. Several Soviet writers have reported that the flora of nonleguminous plant roots could be modified by inoculation with a resulting increase in crop yield. This literature was reviewed by Allison (29) in connection with the work on "azotogen." In view of the negative results obtained by him and his co-workers (30) and by Clark (31), these claims of the Russian workers should be substantiated before they are accepted.

The qualitative nature of the rhizosphere flora was studied by Lochhead (190) and Timonin (191) and earlier observations corroborated that a great difference existed between the flora of the roots and that of the soil. That the rhizosphere flora is affected by the secretions from the roots was established by West (192) and West & Lochhead (193). Thiamine, biotin, and amino acids were secreted and favored the development of those types of microorganisms that had complex nutritive requirements. This was called the "rhizosphere effect" and was noted to be different between resistant and susceptible varieties of flax and tobacco. Timonin (194) ingeniously grew aseptic flax plants in solution and noted that the incidence of pathogenic fungi was lowered and that of the saprophytic increased by the "rhizosphere effect" of the resistant variety. Katznelson & Richardson (195) sterilized soil by steam, chloropicrin, and formaldehyde and then made analyses of tomato roots. The same "rhizosphere effect" was found under those conditions. The root flora of mangels was studied in more detail by Katznelson (196) in manured and unfertilized soil. A striking selective action on the numbers of bacteria, actinomyces, fungi, ammonifying and denitrifying bacteria and protozoa was exerted by the mangel roots. The "rhizosphere effect" was also noted on algae, aerobic cellulose-decomposing bacteria, and anaerobic bacteria.

Factors Limiting the Soil Flora

A few years ago the main factor limiting the soil flora aside from the physical factors was thought to be the protozoa. A great amount of work was done over a period of years which has now dwindled to practically nothing. Very recently, Anscombe & Singh (197) tested the effect of eight micropredators on eighty-seven

strains of common and rare soil bacteria. Three of the predators were amoebae, two were slime molds (*Myxomycetes*), and three were species of myxobacteria. Thirteen of the bacteria were attacked by only one predator and seven were inedible to all predators. There was, therefore, great variation in the ability of the micropredator to digest the bacteria, or in resistance of the latter to digestion. Previously Singh (198) found myxobacteria common in soil. Gram negative bacteria were more often attacked by them than were the gram positive. He also studied the myxomycetes (199) and came to the conclusion that they are soil rather than dung organisms. Raper (200 to 203) had made some time before an exhaustive study of the nature, growth, and development of the slime mold *Dictyostelium discoideum*. This species lent itself readily to pure culture study since the spores could easily be picked free of any contamination. Raper & Thom (204) mixed the myxamoebae of two species of *Dictyostelium* and found that they subsequently segregated and gave rise to sorocarps typical of the two species. It was, however, possible to graft portions of a pseudoplasmodium which had fed on a colorless bacterium to a portion of another pseudoplasmodium which had consumed a chromogenic bacterium (*Serratia marcescens*). A portion of the resulting sorocarp was colored red, depending upon the position of the graft containing the coloring matter residue of the bacterium. As a result of his observations, Raper (200) considered this slime mold capable of appreciably altering the bacteriological flora of decaying vegetation in soils.

The isolation of antibiotic substances produced by pure cultures of soil microorganisms in appropriate media has aroused great interest. The work has been adequately reviewed by Benedict & Langlykke (205) and others. Although directed towards the control of pathogens, one can prophesy that increasing interest will be taken in the function of these and other organisms in their natural habitat. Newman & Norman (206) reported that antibiotic or inhibitive substances were present in subsurface soil which prevented rapid development of introduced organisms. Aqueous extracts were without effect, but alcoholic extracts of the soil were inhibitive. Another case of antibiotic activity was given by Nickell & Burkholder (207). *Azotobacter vinelandii* was greatly reduced in numbers or killed completely by actinomycete cultures during incubation together in mixtures of soil and crop residues.

Soil Microorganisms and Erosion Control

The influence of soil organisms in reducing soil erosion has been studied quite extensively during the past few years. A binding action by *Azotobacter indicum* and fungi was noted by Waksman & Martin (208) and Martin & Waksman (209, 210), the latter finding a difference in aggregation due to the materials decomposed. Bacteria seemed to be responsible for aggregation only as they produced by-products that function as cementing materials, according to Peele (211) and Myers & McCalla (212). This is in line with the results of Pohlman & Nottingham (213) that merely numbers of bacteria and fungi did not correlate with aggregation. McCalla (214) found that the quality of the organic matter added was more important than the quantity. Going farther in the analysis, Martin (215) attributed 50 per cent of the effect of *Cladosporium* to the substances formed whereas in the case of *Bacillus subtilis* 80 per cent of its effect was due to the by-products. Martin (216) also found that the microbial by-products, such as polysaccharides, were attacked by at least one microbe and usually by several. Hubbell & Chapman (217) reported that by-products by themselves did not form aggregates and that when such were formed living organisms were always observed in the structure, and that bacteria, actinomyces, and fungi each formed a distinct type of aggregate.

Actinomyces and Fungi in Soil

For a review of the literature on soil fungi, the reader is referred to Waksman (218). Although not complete and dealing mainly with the work in his laboratory, it may serve as a basis of references. During the past decade, perhaps the most interesting work was that of Waksman, Umbreit & Cardon (219) on the thermophilic actinomyces and fungi in soil and composts. Mention should also be made of the classification of the actinomyces by Waksman & Henrici (220).

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BIOLOGICAL NITROGEN FIXATION[1]

By Artturi I. Virtanen
Biochemical Institute, Helsinki, Finland

In the current decade both monographs and reviews have been published on the biological nitrogen fixation. The most important among them are the monograph by Wilson (1) on symbiotic nitrogen fixation, the reviews by Burk & Burris (2), and Burris & Wilson (3) on biological nitrogen fixation. In a critical review Virtanen (4) has recently dealt with the biology and chemistry of nitrogen fixation by legume bacteria and Wilson & Burris (5) have discussed the mechanism of biological nitrogen fixation. Tóth (6), in his monograph, has treated symbiotic nitrogen fixation, especially in animals, and Fogg (7), in his short review, has discussed nitrogen fixation by blue-green algae. The above works cover the literature up to 1947. In the present paper I shall therefore restrict myself to examining the most discussed problem of the mechanism of nitrogen fixation in the light of the recent investigations, to reporting some new observations regarding nitrogen-fixing organisms, nitrogen metabolism in plants and microorganisms, and, further, to reviewing some topics omitted in the earlier reviews most widely read.

Mechanism of Nitrogen Fixation

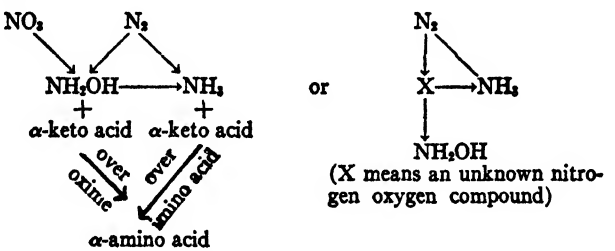
Definite information is not available concerning the initial stages of nitrogen fixation. In the nitrogen fixation under aerobic conditions, which has been almost exclusively examined in greater detail (leguminous root nodules and *Azotobacter*), small amounts of oxime nitrogen are formed. On the basis of this the occurrence of hydroxylamine in nitrogen fixation is very probable [cf. Virtanen (4, 8)]. According to Virtanen (9), the first step in nitrogen fixation can be presumed to be ionization of nitrogen: $N_2 \rightarrow N_2^+$; the energy of ionization is 16.7 eV (105). The reactions: $N_2 \rightarrow 2N$ (11.4 eV) and $N \rightarrow N^+$ (14.5 eV) may possibly come into question. Transfer of electrons may be affected by the iron catalyst system just as in respiration. The inhibition of nitrogen fixation by carbon monoxide (10 to 14) could then be attributed to the formation of the $Fe^{++}CO$ complex (4), and the inhibition by H_2 (1, 10, 15, 16, 17) can be

[1] This review covers the period from approximately January, 1947 to March, 1948.

attributed to the competition between nitrogen and hydrogen (9) because the energy of ionization of hydrogen is smaller than that of nitrogen [$H_2 \rightarrow H_2^+$ (15.4 eV); $H_2 \rightarrow 2H$ (4.4 eV); $H \rightarrow H^+$ (13.5 eV)]. Transfer of electrons from nitrogen to oxygen would result in nitrogen oxide. According to this concept hydroxylamine can be expected to arise at a certain stage of reaction ($N_2 \xrightarrow{O_2,\ H_2} NH_2OH$). Hydroxylamine could be further reduced to ammonia, or it might react with keto acids.

Another mechanism for nitrogen fixation, reduction of nitrogen to ammonia (e.g., $N_2 \rightarrow 2NH \rightarrow 2NH_2 \rightarrow 2NH_3$) would correspond to the technical synthesis of ammonia. Formation of hydroxylamine can be explained even when the reaction proceeds this way by assuming the reaction $NH_2OH \rightarrow NH_3$ to be reversible (3). Oxidation of ammonia to hydroxylamine has, however, not been noted with *Azotobacter*, legume bacteria, or with a *Torula*-yeast (cf. below).

Since on the reduction of nitrate small amounts of oxime nitrogen arise (cf. below), as in the fixation of nitrogen, the mutual relations of N_2, NO_3^-, and NH_4^+ in the amino acid synthesis can be illustrated by the scheme:



Burris & Wilson (3) have assumed in their scheme, introduced in 1945, that nitrogen fixation and reduction of nitrate occurs quantitatively over hydroxylamine, this being further reduced to ammonia. In their most recent paper [Wilson & Burris (5)] they no longer consider hydroxylamine indispensable for the formation of ammonia from N_2.

In the publications concerning the mechanism of nitrogen fixation chief attention is drawn to the question whether hydroxylamine or ammonia is formed as the particular product of fixation which together with a carbon compound produces the first organic nitrogen compound. This question is closely associated with the

problem of the identity of the primary amino acid and the mechanism of its formation.

Concerning the formation of amino acids in nitrogen fixation, Virtanen & Laine (18) first advanced the hypothesis, based on the excretion products of the root nodules, that the amino dicarboxylic acids, especially aspartic acid, are primarily formed. The transamination observed by Braunstein & Kritzman (19, 20) in animal tissues, and also noted with green plants and root nodules (21, 22), explains the easy interconversion of aspartic and glutamic acids. The formation of alanine from amino dicarboxylic acids can also be explained by transamination, possibly also that of the higher homologues of alanine although the reaction velocity then is small (23). Formation of aromatic amino acids via transamination has not been observed in plants and the question of their mode of formation is open.

The occurrence of amino dicarboxylic acids as the primary amino acids was later shown to be independent of the nature of the nitrogen source. Several investigations with animals, plants, and microorganisms [Foster *et al.* (24); Schoenheimer *et al.* (25); and Vickery *et al.* (26)] demonstrated that N^{15} in compounds used as nitrogen sources has a tendency to accumulate particularly in amino dicarboxylic acids. The studies by Roine (27) in this laboratory with *Torula*-yeast showing that the low-nitrogen yeast rapidly takes up ammonium salts in aerated conditions and forms at first soluble nitrogen compounds containing only amino dicarboxylic acids, their amides, and alanine, but no other amino acids speak strongly in favor of the formation of amino dicarboxylic acids as the primary amino acids. According to Virtanen & Csáky (28) *Torula*-yeast forms similar amino acids from nitrates as from ammonium salts. Independent of the nitrogen source (N_2, NH_4^+, NO_3^-, amino acids, etc.), the amino dicarboxylic acids would thus arise as the primary amino acids.

Burris (29) and Burris & Wilson (30) have supplied *Azotobacter* in experiments of short duration with N^{15} as either N_2 or NH_4^+ and have noted in both cases that N^{15} accumulates in the same fractions, notably glutamic and aspartic acids. The highest level of isotopic nitrogen was found in the glutamic acid fraction. These investigations lead thus to the same result as the above mentioned: amino dicarboxylic acids are likely to be the primary amino acids, independent of the nitrogen source.

A final conclusion as to which of the two amino dicarboxylic acids, glutamic or aspartic, is the primary one in nitrogen metabolism is rendered difficult by the existence of the transamination reaction, the velocity of which it is not easy to determine in the living cell. In the light of the information now available on the enzymes participating in amino acid synthesis some conclusions can, however, be made.

A specific glutamic acid dehydrogenase [Euler *et al.* (31, 32) and Adler *et al.* (33, 34)] catalyzing the reaction

$$HOOC \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \rightleftarrows HOOC \cdot (CH_2)_2 \cdot C(=NH) \cdot COOH \rightleftarrows HOOC \cdot (CH_2)_2 \cdot CO \cdot COOH + NH_3$$

which seems to occur commonly in living cells explains the formation of glutamic acid from ammonia and α-ketoglutaric acid. A corresponding enzyme has not yet been found for aspartic acid. True, by means of the enzyme aspartase (35, 36, 37), aspartic acid is produced from ammonia and fumaric acid, and in many bacteria with ample aspartase this mode of formation of aspartic acid is plausible. Nevertheless, aspartase can be found in green plants only at the seedling stage (36) and, as a rule, not at all in animal organisms. In root nodules and legume bacteria, aspartase is likewise not to be found (18). In general, glutamic acid is therefore the most plausible primary amino acid when ammonia serves as the nitrogen source, and insofar as nitrogen fixation leads to ammonia, also when N_2 forms the nitrogen source. Recently, Stokes, Larsen & Gunnes (38) have reported on participation of biotin in aspartic acid synthesis in certain microorganisms. This suggests that after all in the synthesis of aspartic acid from ammonia and oxaloacetic acid there might also act a specific enzyme other than aspartase and transaminase which are not affected by biotin. The role of biotin in the aspartic acid synthesis is, however, not yet known in detail; accordingly, the suggested possibility is only a surmise. The accumulation of aspartic acid in the substrate of inoculated legumes does not per se speak against glutamic acid as a primary amino acid, because transamination and the use of glutamic acid for protein synthesis may cause the accumulation. In any case it is peculiar that only once a small amount of glutamic acid was found among the excretion products in addition to aspartic acid [Virtanen *et al.* (39)].

Rautanen (40, 41) has in this laboratory examined the nitrogen

metabolism of higher plants using pea as a test plant. He allowed cut plants to suck up different nitrogen compounds in aqueous solutions and determined the composition of the soluble nitrogen fraction of the plants after an interval of a few hours. According to these experiments, glutamic and aspartic acids are the first to accumulate in the soluble nitrogen fraction. Also their amides and to some extent alanine are to be found. The situation thus resembles that in yeast. The results do not give any indication as to which of the amino dicarboxylic acids arises first. When the cut plants are given aspartic acid, the total amount of aspartic acid plus asparagine in the soluble nitrogen fraction does not increase much in five hours, whereas the sum of glutamic acid plus glutamine markedly rises. After eleven hours the sum of aspartic acid plus asparagine is still unchanged, while that of glutamic acid plus glutamine has greatly increased. In alanine a steady slow increase is noted. On the other hand, feeding with glutamic acid raises the sum of glutamic acid plus glutamine already in five hours, but leaves that of aspartic acid plus asparagine entirely unaffected. Aspartic acid accordingly appears to be converted more rapidly in plants than glutamic acid.

The observation of Burris (29) and Burris & Wilson (30) on the accumulation of N^{15} mostly in glutamic acid during nitrogen fixation gives support to the concept that this amino acid is formed primarily and that consequently ammonia is the nitrogen compound used in amino acid synthesis (5). Aspartic acid would be formed from glutamic acid by transamination. Several other observations with different organisms point in the same direction. However, since the velocities of different reactions in the living cell are unknown, even this evidence is unconvincing (cf. above). It must be taken into account that several lactic acid bacteria are unable to synthesize glutamic acid and for them glutamic acid is therefore an indispensable nutrient [cf. Snell (42)]. The same relations are also found with aspartic acid in certain lactic acid bacteria.

Another and probably the most weighty evidence of Burris & Wilson (30) and Wilson *et al.* (43) for the formation of ammonia as a result of nitrogen fixation is that ammonia is completely and immediately accepted as a source of nitrogen to the exclusion of the nitrogen fixation reaction independently of whether the organism has been cultivated earlier on N_2, NH_4^+ or NO_3^-. Compounds

readily converted into ammonia, as urea, also cause complete inhibition of fixation. Compounds less readily converted into ammonia, as nitrate or nitrite, require a period of adaptation before they effectively inhibit fixation. Also in this case ammonia is considered to be the inhibitory nitrogen compound.

According to Virtanen *et al.* (44) the nitrogen fixation in leguminous root nodules is affected by ammonia and nitrate in a contrary way to that in *Azotobacter*. The fixation is not completely inhibited by concentrations of 25 to 100 mg. ammonia nitrogen per liter of nutrient solution, although it is naturally lessened while the plants take a part of their nitrogen nutrition through the roots. Calcium nitrate, on the contrary, inhibits nitrogen fixation even at lower concentrations. A quantity of 25 mg. nitrate nitrogen per liter of nutrient solution prevented fixation in most experiments, and 50 mg. in all cases. (Because of nitrogen loss in growth detection of a weak nitrogen fixation in plants is often impossible without the use of a nitrogen isotope.) However, it should be pointed out that the inhibitory effect of nitrate on nitrogen fixation in nodules influences the formation and size of nodules and probably the hemoglobin of the nodules (cf. below) and is thus not directly comparable with the corresponding effect on the *Azotobacter*.

Numerous experiments performed by Virtanen & Linkola (45) have shown that the pea takes up ammonium nitrogen from the nutrient solution more rapidly than nitrate nitrogen. Since, however, ammonium nitrogen has a much weaker effect on nitrogen fixation than nitrate nitrogen, the hypothesis [cf. Wilson (1)] that the observed inhibitory effect of nitrate nitrogen on nitrogen fixation depends on an excessive nitrogen-carbohydrate ratio in the plants cannot explain the inhibition. Obviously, the question then concerns in the first place the specific effect of nitrate nitrogen possibly on leghemoglobin (e.g., the formation of nitrite-hemoprotein compound) [Virtanen (4)].

Oxime nitrogen has been found in nitrogen fixation as an excretory product both with *Azotobacter* (46) and with inoculated legumes (8, 47). Besides, when *Azotobacter* grows on nitrate, oxime nitrogen is detected in the nutrient solution (48). In 1947–48 we studied in this laboratory the formation of oxime nitrogen with *Torula*-yeast, legume bacteria, and *Azotobacter* by suspending the microorganisms in a nitrate or ammonium salt solution and estimating oxime nitrogen in the cell mass. After different experi-

mental periods the mass of microorganisms was separated and suspended in 8 per cent trichloroacetic acid solution. From the suspension oxime nitrogen was determined according to Blom by boiling first for six hours with 3 N H_2SO_4 solution. Nitrite, hyponitrite, and nitrohydroxamic acid do not interfere with the determination, because they are quantitatively decomposed in the sulfuric acid solution during boiling. Virtanen & Csáky (28) have found that both the low-nitrogen and normal-nitrogen *Torula*-yeasts suspended in an aerated nitrate solution rapidly form oxime nitrogen in their cells. The maximum amount is attained in ten to fifteen minutes. With ammonium sulphate no traces of oxime nitrogen are detectable. With normal nitrogen legume bacteria similar results have been obtained as with *Torula*. The maximum amount of oxime nitrogen was then attained in one to two hours. With *Azotobacter* the oxime nitrogen reached a maximum in two to three hours. When suspended in ammonium sulfate solution neither of the bacteria formed oxime nitrogen (49). Oxidation of ammonia to hydroxylamine has, accordingly, not been established with nitrogen-fixing bacteria nor with *Torula*-yeast. Only with strongly oxidizing molds has Steinberg (50) observed formation of oxime nitrogen both in nitrate and ammonium salt solutions.

Virtanen (4, 51) believes that the only weighty reason to doubt the participation of hydroxylamine in amino acid synthesis is the fact that no enzymes are known to catalyze the reaction between hydroxylamine and α-ketoglutaric acids. If the reaction is purely chemical, it may result in several different oximes since hydroxylamine reacts readily not only with oxaloacetic acid, pyruvic acid, and α-ketoglutaric acid, but also, e.g., with ascorbic acid. As the amounts and mutual relations of the keto substances vary in plants, for instance, depending on illumination, the nonenzymatic reaction between hydroxylamine and keto substances is not controlled. If this be true, the synthesis of amino acids over oximes evidently cannot be an important reaction, although the formation of oxime nitrogen in the nitrogen fixation implies that such a synthesis takes place to a certain extent.

Information on the suitability of hydroxylamine for the nitrogen nutrition of cells as well as on its participation in enzymatic reactions is, however, very deficient. In general, hydroxylamine and oxime were earlier held unsuitable as nitrogen sources and this was regarded as an important argument against hydroxylamine as

an intermediate in nitrogen fixation. The latest observations on the participation of hydroxylamine in enzymatic reactions and on its suitability for the nitrogen metabolism of cells indicate, however, that hydroxylamine is not so strange for the organisms as is commonly reported in the literature. Steinberg (50) has shown that *Aspergillus* uses hydroxylamine in low concentrations for its nitrogen nutrition and produces the same amount of cell substance per unit weight of hydroxylamine used as with ammonia or nitrate.

Virtanen & Csáky (28, 49) noted that *Torula utilis* grows on the surface of agar with hydroxylamine as its sole nitrogen source. The growth improved when the hydroxylamine concentration was raised from 1:25000 to 1:5000, provided that the yeast was grown first in the former concentration and then transferred to the latter. In the concentration 1:1000 the growth was already weaker. In nutrient solutions *Torula* did not grow in the above hydroxylamine concentrations.

Elliot (52) and Elliot & Gale (53) report that the enzyme system synthesizing glutamine from glutamic acid and ammonia forms hydroxamic acid when the ammonia is replaced by hydroxylamine (glutamic acid, adenosinetriphosphate, and hydroxylamine in the presence of the enzyme system). Lees & Quastel (54) have noted that pyruvic oxime (0.005 *M*) is nitrified with great rapidity by a bacteria-saturated soil and does not inhibit the conversion of added NH_4^+ to NO_3^-. The presence of sodium pyruvate (0.005 *M*) entirely eliminates the toxic effect of hydroxylamine (0.005 *M*) and allows full nitrification of the hydroxylamine to take place. They discuss the possibility that pyruvic oxime and other analogous molecules have a great significance in the process of nitrification. Yeast reduces oximes to the corresponding amines. Maurer (55) has noted that pyruvic oxime is reduced by yeast to alanine. Hydroxylamine is thus not so "unnatural" a nitrogen compound as has been presumed. This fact does not per se prove that hydroxylamine is an intermediate. New findings reveal, in any case, that hydroxylamine may replace ammonia at least in one enzyme system and that, accordingly, it is not impossible that it may function in some others as well.

It seems that the initial stages of nitrogen fixation cannot be clarified until more light is obtained on the corresponding enzyme system in the nitrogen-fixing organisms. Information concerning

this is entirely lacking. The much discussed participation of molybdenum in nitrogen fixation seems to acquire great significance in the elucidation of the process. Bortels (56) established first the indispensability of molybdenum for maximum nitrogen fixation by *Azotobacter*. This observation has been confirmed by many investigators and it has been extended to other nitrogen-fixing organisms and symbiotic fixation in leguminous root nodules. In recent years Anderson (57, 58), in particular, has supported the idea that molybdenum is essential for symbiotic nitrogen fixation. On the other hand, Steinberg (59, 60) has shown that the requirement for molybdenum is evident when *Aspergillus* grows on nitrate nitrogen. With ammonium nitrogen the response to molybdenum was found to be considerably less. Mulder (61) has confirmed this finding. He has notably widened our knowledge of the role of molybdenum. He regards molybdenum as indispensable for denitrifying bacteria. All observations on molds, denitrifying bacteria, and green plants show that molybdenum is acting as a catalyst in nitrate reduction. *Azotobacter chroococcum* needs molybdenum when growing on nitrate nitrogen and gaseous nitrogen. With ammonium sulfate no response to molybdenum could be observed at all. Nitrogen fixation by the leguminous root nodules likewise greatly suffers from the lack of molybdenum. These observations seem likely to support the idea of nitrogen fixation through oxidation and to explain the significance of molybdenum for nitrogen fixation. True, Mulder (61) concludes from the fact that the molybdenum requirement is greater when *Azotobacter* grows without combined nitrogen than with nitrate nitrogen that the molybdenum-requiring reactions involved in nitrate and N_2 assimilations are not similar, but by analogy I think there is every reason to assume that molybdenum catalyzes reduction reactions in both cases. Woods (62) has demonstrated that washed suspensions of *C. welchii* and *E. coli* are able to catalyze the reductions of nitrate, nitrite, and hydroxylamine to ammonia by molecular hydrogen, that nitrite is an intermediate in the reduction of nitrate, and that there is some evidence that hydroxylamine may be an intermediate in the further reduction of nitrite. After the role of molybdenum at different stages of reduction is disclosed it is possible that new light will simultaneously be obtained on the process of nitrogen fixation. The findings of Steinberg (50) with

Aspergillus niger have already shown that molybdenum exerts a powerful effect on the reductions of nitrate, nitrite, and nitrohydroxamic acid, but not on hydroxylamine.

Although nothing certain can be said of the initial stages of nitrogen fixation, recent progress, nevertheless, has sided in making the problem more condensed and precise. The formation of oxime nitrogen in aerobic nitrogen fixation is a fact not to be overlooked. The oxime found by us in *Torula*-yeast, *Azotobacter*, and legume bacteria is presumably a comparatively stabile compound which only slowly disappears in the metabolism of cells. Its detection, however, makes the formation of hydroxylamine evident and it remains in the first place to examine how important a role hydroxylamine plays in nitrogen metabolism. Ammonia may well be the nitrogen compound which as a final product of nitrogen fixation combines with α-ketoglutaric acids. Even then the problem of greatest importance will still be to solve how the initial stages of nitrogen fixation take place and in what way ammonia arises. For instance, nitrohydroxamic acid which according to Steinberg (50) is a comparatively good nitrogen source for *Aspergillus* may be one of the intermediates in nitrogen fixation. However, speculations on the formation of oxygen compounds of nitrogen lack experimental support.

Hemoglobin in Root Nodules

Kubo (63) was the first to identify the red pigment in leguminous root nodules as a hemoprotein which is able to take up and give off molecular oxygen. The pigment belonged thus to the hemoglobins. Burris & Haas (64) failed at first to establish the hemoglobin character of the substance, this obviously because the iron of the pigment is easily oxidized to trivalent. The communication next in order was by Virtanen (65, 66) who confirmed the hemoglobin nature of the pigment and showed at the same time that the pigment is essentially associated with the nitrogen-fixing ability of the nodules. The nodules formed by ineffective bacterial strains do not contain hemoglobin at all. In effective nodules, on the other hand, hemoglobin is invariably found. The pigment was thus for the first time on an experimental basis brought into connection with the nitrogen fixation. Keilin & Wang (67) also corroborated the observation of Kubo on the hemo-

globin nature of the pigment, and so did Little & Burris (76) later. Virtanen & Laine (68) have suggested for the sake of brevity the name leghemoglobin for the hemoglobin of root nodules. When

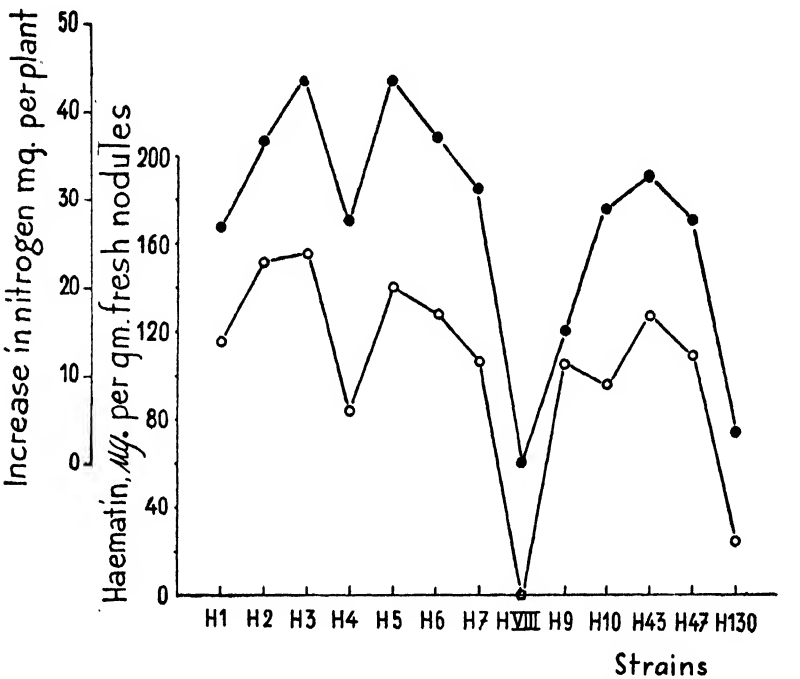


FIG. 1.—Hematin content of the nodules of Torsdag pea inoculated with different bacterial strains. Period of growth: April 23 to June 17, 1947. Plants in full bloom at the end of the period, nodules chiefly red, a few green nodules in some experiments. The low absorption of inactive H VIII nodules was subtracted from each experiment.

the pigment is mentioned in connection with root nodules, hemoglobin can of course be used.

Virtanen *et al.* (44, 69) and Virtanen (70) have more closely examined the correlation between nitrogen fixation and the hemoglobin content of nodules with numerous bacterial strains of pea and horse bean. The concentration of the pigment was determined by dissolving the hematin from the crushed nodules in pyridine and by determining the absorption at 530 mμ. In fully

inactive nodules hemoglobin cannot be spectrophotometrically detected and their very low hematin content is due to other hematin compounds (cytochromes, etc.). The insignificantly small hematin content of inactive nodules indicates that the high hematin content of the effective nodules belongs chiefly to hemoglobin. On comparing the hematin concentration of nodules formed by thirteen different pea Rhizobium strains with varying activity to the nitrogen fixation in pea plants inoculated with the respective strains a positive correlation can be noted between the hemoglobin content of the nodules and the intensity of the nitrogen fixation (Fig. 1). Similar results have been obtained with the horse bean. The hematin content of the nodules of pea, expressed as μg. hematin per gm. fresh nodules, has in our experiments varied from 20 to 180 μg. depending on the efficiency of the bacterial strain.

The red pigment of the effective nodules gradually changes to green with the advance of growth (44, 66, 68, 70). When the nitrogen fixation has ceased all the nodules are green. This can be demonstrated best in the sterile culture system in transparent flasks. Also in pot cultures in quartz sand the change of color is detectable. Under natural conditions the green nodules are preserved well enough to be easily visible if the soil is dry. In wet soil, however, the older nodules are rapidly decomposed by foreign microorganisms and therefore it is difficult to discern green nodules. Removal of inoculated plants in full growth into dark for a few days also causes the nodules to turn green. The change takes place more rapidly on pea than on soybean. The essentiality of leghemoglobin for nitrogen fixation is confirmed also by these observations on the cessation of fixation at the change of the color of the pigment from red to green.

In experiments in a sterile culture system with inoculated pea plants Virtanen & Erkama (71) have studied the significance of iron and copper to the formation of nodules and leghemoglobin. The iron content of the nutrient solution has then a great bearing on the hemoglobin content. Likewise, copper seems to be indispensable. The investigations are still of a preliminary nature.

Leghemoglobin is easily oxidized to the met-form (66, 68). After crushing and centrifugation of the nodules, the solution often contains exclusively methemoglobin. The oxidizing ability of the

nodules varies considerably judging from the fact that in certain instances leghemoglobin is retained in the crushed nodules much better than in others although the outward conditions are the same. The great ease of oxidation of leghemoglobin may be due either to a strong phenol oxidase in the nodules [Keilin & Wang (67); Virtanen (4)] or to the easy autoxidation of leghemoglobin [Keilin & Wang (72)], or to both factors. The experiments in which hemoglobin of blood was added to a mass of excised ineffective hemoglobin-free strain H VIII nodules (see Fig. 1), which then were crushed, show that even this markedly stabile hemoglobin is rapidly oxidized to methemoglobin by the oxidase of the nodules [Virtanen & Sternberg (73)]. So the rapid oxidation of leghemoglobin in crushed nodules does not per se prove that it is autoxidized with greater ease than the hemoglobin of blood.

Whether methemoglobin is present in intact nodules is a question in dispute. Virtanen & Laine (68) consider that both hemoglobin and methemoglobin are found in nodules, their mutual ratio varying with light intensity, age of plants, and possibly other factors. This idea was based on the determination of the absorption band from solutions obtained from crushed nodules after addition of sodium fluoride. Although the nodules were crushed as rapidly as possible at a low temperature and then quickly centrifuged, methemoglobin was, nevertheless, invariably found in the nodule extract. True, the oxidase may induce oxidation of leghemoglobin during the treatment. Keilin & Smith (74) failed to find methemoglobin when examining crushed or sliced nodules directly without bringing the pigment into solution. They paid particular attention to the absorption of methemoglobin at about 625 mμ. This maximum is, however, weak at the natural pH of nodules; therefore its detection is difficult in crushed or sliced nodules, unless the quantity of trivalent iron is high. In some experiments, however, they found even after addition of sodium fluoride only a weak maximum pointing to methemoglobin. In our experiments after the addition of sodium fluoride to the crushed nodules the absorption maximum of the fluorine compound of methemoglobin at 605 to 610 mμ was in general the more distinct the deeper the brownish tinge of the nodules. Unfortunately, a comparable determination cannot be made with intact nodules and therefore no convincing evidence is available of the existence of methemoglobin in them.

Purification of leghemoglobin by repeated precipitation with ammonium sulfate has led to a preparation whose iron and hemin content is the same as that of the hemoglobin of blood [Virtanen *et al.* (44)]. With this preparation Pedersen in Upsala has found by the ultracentrifuge method a molecular weight of about 17,000 (69). With the earlier preparations whose degree of purity judged from the hematin content was about 80 to 85 per cent Pedersen obtained the value of 34,000 (75). Which of these values represents the real particle size of leghemoglobin cannot be said for certain. Possible dissociation of leghemoglobin at some pH levels has not yet been examined.

Hemoglobin in root nodules is located in the plant cells outside the bacteroids. Virtanen *et al.* (44) arrive at this conclusion on the basis of the fact that the pigment passes easily and quantitatively into solution on crushing of nodules and that the bacteroids are then not much broken. Keilin & Smith (74) also hold the view that the pigment is located in the plant cells where the bacteria are found. The nodules just formed do not yet contain hemoglobin and their bacteria are then rod-shaped. Within a few days red pigment begins to form and simultaneously the rods are transformed into bacteroids [Virtanen (65, 66); Virtanen *et al.* (44)]. The effective red root nodules of pea contain almost exclusively bacteroids (4, 44, 65, 66). When the red pigment of nodules turns green and nitrogen fixation ceases, the bacteroids disappear for the most part and only rods are found in the nodules (44). How the rods arise in the green nodules remains still to be determined. The ineffective white nodules always contain rods only (44, 65, 66).

Hemoglobin has not been found in *Azotobacter* nor in the root nodules of alder (51). It therefore seems to be essential only for the root nodules of the legumes. It can be proved to function as a store and carrier of oxygen. Kubo (63) noted that the addition of the nodule-pigment to a culture of *Rhizobium* stimulated respiration. The same observation was made by Little & Burris (76) at a low pO_2. Virtanen (68) has, in addition, speculated on the possibility that the valency change of iron taking place in leghemoglobin would be significant for nitrogen fixation, but no evidence is provided in support of this hypothesis.

The attempts to accomplish nitrogen fixation with excised root nodules have partly led to positive results. In addition to the

results obtained in this laboratory (4), Burris *et al.* (77) and Wilson & Burris (5) have succeeded in demonstrating nitrogen fixation in many experiments by using heavy nitrogen, though not regularly. With excised nodulated roots the results were at first always positive. In their latest paper, Machata, Burris & Wilson (78) report that the results are variable even with excised nodulated roots. Of the experiments carried out by the Wisconsin group in the course of five years with 133 samples of nodules 16 per cent have given positive results if one adopts the statistically significant gain of 0.05 atom per cent N^{15} excess as the criterion. They attribute the positive results to possible infections by foreign bacteria. Although it is impossible to completely avoid contaminations, the best results in favor of fixation, both in Wisconsin and in Helsinki, are difficult to explain as being due to nitrogen fixation by foreign bacteria. A copious nitrogen fixation observed in certain experiments during twenty to twenty-four hours at room temperature can hardly be ascribed to contamination, the less so as Machata *et al.* have performed some of the experiments with nodules initially free from foreign microorganisms. It seems most likely that deteriorative changes in the machinery required for nitrogen fixation take place in different lots of excised root nodules at different speeds and consequently the results are variable.

With free-living legume bacteria no nitrogen fixation could be accomplished even in the presence of leghemoglobin [Virtanen, (44, 75); Wilson & Burris (5)]. As stated above leghemoglobin is rapidly oxidized in crushed nodules to legmethemoglobin and does no longer function as a carrier of oxygen. Building up a complete system for nitrogen fixation with free-living legume bacteria seems impossible as long as such a result has not been attained even with excised root nodules.

Nitrogen Fixation by Blue-Green Algae

The first observation on nitrogen fixation by pure cultures of blue-green algae was made by Drewes (79) in 1928. Later the pure culture techniques have been developed especially on the basis of the findings of Allison & Morris (80) and Bortels (81) which rendered it possible to destroy the bacteria in the cultures of blue-green algae by means of ultraviolet light. De (82) and Fogg (83) have particularly emphasized the necessity of the purity of

the cultures, and it can now be held conclusively proved that at least many species of *Notocaceae* fix atmospheric nitrogen. This has also been demonstrated recently by means of the heavy isotope of nitrogen [Burris *et al.* (77)].

Nitrogen fixation by blue-green algae is especially interesting in that these organisms assimilate carbon dioxide and are thus independent of organic carbon compounds and of combined nitrogen as well. Consequently, they are the most completely autotrophic organisms known. Light is then indispensable for them. Winter (84), Allison & Hoover (85), and De (86) have shown that some species of blue-green algae are able also to grow and fix nitrogen in the dark in the presence of suitable carbohydrates. According to Allison & Hoover (85) growth and nitrogen fixation are increased by sugars even in light.

The blue-green algae fix nitrogen only in very poor conditions because nitrates and ammonium salts prevent fixation as Fogg (83) has found. An acid reaction is unfavorable for fixation, this ceasing below about pH 5.7 (85, 86). The optimum is at a slightly alkaline reaction (pH 7 to 8.5). Nitrogen fixation is associated with growth. Burris & Wilson (88) have shown that in blue-green algae the half-maximum rate of fixation occurs at a partial pressure of nitrogen of the same order of magnitude as with *Azotobacter* and leguminous root nodules. Carbon monoxide and hydrogen, too, inhibit nitrogen fixation in blue-green algae. The mechanism of nitrogen fixation and the enzyme system in them are obviously similar to those in *Azotobacter* and leguminous root nodules.

The dependence of the blue-green algae on light, as well as their inability to fix nitrogen in the presence of nitrate, makes their role in agriculture significant only for ricefields [De (82), De & Bose (89), and Singh (90)], where the soil is waterlogged, and the action of nitrogen-fixing bacteria is probably unimportant. Blue-green algae are frequently met in abundance in fresh water, especially in neutral and alkaline waters with a relatively high content of dissolved organic matter and a low content of nitrate [Pearsall (91)]. In such waters the algae have been reported to be the chief agents responsible for increases of combined nitrogen (85). In addition to free-living forms there are many blue-green algae living in association with other plants. Winter (84) has with great probability proved the nitrogen fixation in such cases. He has also

found that a species of algae, *Nostoc punctiformis*, isolated by him from cycads, hardly can live autotrophically. In certain cases the host plant is able to grow without combined nitrogen by means of its symbiotic algae. This was observed by Bortels (81) with the water fern *Azolla*. In general, the symbiosis seems to be rather weak and either partner can live independently of the other although the nutrition requirements may then differ from those in symbiosis. On bare rocks the nitrogen-fixing algae are able to grow and thus induce vegetation even on them [cf. Fogg (92)].

Symbiotic Nitrogen Fixation in Insects

Pekló (93) was the first to advance the assumption that the bacteria living in symbiosis with plant-lice fix atmospheric nitrogen. According to his investigations the particular microorganisms belong to the *Azotobacter* group. Pekló did not experimentally prove nitrogen fixation. Buchner (94) also presumed symbiotic nitrogen fixation to occur in plant-lice as well as leguminous root nodules. Schoel (95) was unable to furnish experimental proofs for the nitrogen fixation by plant-lice but nevertheless held it possible. Gropengiesser (96) claimed to have accomplished weak nitrogen fixation with bacteria isolated from *Periplaneta orientalis*.

Cleveland (97) examined the possibility that termites might grow exclusively on cellulose and arrived at the result that "the termites must be able in some way to fix atmospheric nitrogen which they use in manufacturing proteins." But his direct experimental proving does not bear criticism. Tóth (98) and Tóth & Wolsky (99) determined the respiration quotient in plant-lice and noted it to be considerably lower than one (0.86). On the basis of this they concluded that the proteins participate effectively in the metabolism of plant-lice although the plant sap chiefly contains carbohydrates and very little nitrogen-containing substances. The plant-lice must thus get nitrogen from some other source than food. Rapidly multiplying plant-lice lose daily nitrogen to the extent corresponding to the total protein content of their body, consequently, their nitrogen supply must be considerable.

Tóth, Wolsky & Batóri (100) succeeded in experimentally demonstrating nitrogen fixation by plant-lice by adding oxaloacetic acid to crushed abdomina of plant-lice, in the similar manner as Virtanen & Laine had treated leguminous root nodules. Later Tóth

(6) noticed that oxaloacetic acid could be replaced by succinic acid. The intensity of the nitrogen fixation is indicated by the following results with the crushed abdomina of *Pterocallis juglandis:* with oxaloacetic acid, at the beginning of the experiment, nitrogen 0.287 mg., after 24 hr. 0.716 mg; without oxaloacetic acid, at the beginning of the experiment, nitrogen 0.343 mg., after 24 hr. 0.367 mg. Schanderl (101) isolated a yeast symbiont from the beetle *Rhagium inquisitor* which during a period of slightly over two months fixed in its cultures detectable amounts of nitrogen (nitrogen gain about 23 per cent). Tóth, Wolsky & Bátyka (102) obtained with a mixed culture of short rods and cocci isolated from *Aphrophora salicis* in ninety-six hours a 67 per cent gain in the fixed nitrogen when the nutrient solution contained oxaloacetic acid. They consider that essential factors in nitrogen fixation are: the symbiotic bacteria, oxaloacetic acid, and probably an unknown substance produced by the host animal.

According to Tóth the symbionts of plant-lice resemble *Azotobacter.* Peculiarly enough, they do not fix nitrogen in sugar solutions to any marked extent, unless supplied with oxaloacetic acid. Succinic and citric acids can substitute for oxaloacetic acid. The indispensability of C_4-dicarboxylic acids in the nutrient solution wants more clarification.

The existence of symbiotic nitrogen fixation has already been claimed in numerous species of insects: four species of *Aphididae,* ten of *Homoptera,* seven of *Heteroptera,* one of *Aleurodidae,* one of *Isoptera* (103), and one of *Blattaria* [Tóth (6)].

Establishment of symbiotic nitrogen fixation in the animal kingdom is apt to open new aspects to research. In this field, however, much additional clarification is needed since many data reported in the literature do not possess a very great power of evidence.

Antibacterial Effect of Soil Microorganisms on Nitrogen-Fixing Bacteria

Observations on the problem covered by the title are not found in the earlier literature. Recently, however, Virtanen & Linkola (104) made the observation that *B. mesentericus* effectively prevents the growth of legume bacteria and *Azotobacter.* In some autoclaved samples of soil rich in organic matter inoculation with legume

bacteria could not induce any growth. A closer examination revealed that a bacterium of *B. mesentericus* type isolated from the samples has an antibacterial effect upon all tested strains of rhizobia from pea, clover, and *Phaseolus*, as well as on *Azotobacter*. The only exception was a strain of soybean *Rhizobium* which grew quite unaffected (other strains of soybean *Rhizobium* were not available). On different strains of pea *Rhizobium* the influence of the bacillus was also slightly variable. On many soil bacteria tested the bacillus had no antibacterial effect. Also *Staphylococcus* and *E. coli* grew well in the vicinity of the bacillus colony. Two strains of *B. subtilis* used had no distinct antibacterial effect on the rhizobia.

The growth-preventing substance excreted by *B. mesentericus* is soluble in ether. Its examination is just in progress. The ether extract contains *interalia* volatile fatty acids. The antibacterial substance is chiefly found in this fraction. It should be mentioned that *n*-butyric and valeric acids, isobutyric and valeric acids, caproic acid, and caprylic acid also inhibit the growth of legume bacteria.

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