

PERSPECTIVES  
AND HORIZONS  
IN *Microbiology*





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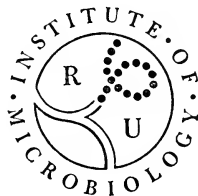


Perspectives and Horizons  
in MICROBIOLOGY



PERSPECTIVES  
AND HORIZONS  
IN **Microbiology**

A SYMPOSIUM EDITED BY  
Selman A. Waksman



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## Preface

Microbiology has made great strides in the last eight or nine decades. Beginning with studies of microorganisms as causative agents of disease and fermentation, their occurrence in soil and other natural substrates, and life cycles of certain groups of bacteria and fungi, microbiology has grown into a broad science with numerous theoretical and practical applications. This is true of ecology and taxonomy of microorganisms, physiology and biochemistry, genetics and cytology, and applications to virtually every aspect of human endeavor.

This symposium, arranged in connection with the dedication on June 7, 1954, of the Institute of Microbiology, Rutgers University, presents an attempt to analyze the present and possibly some of the future aspects of microbiology. Who would have thought, in the days of Pasteur and Koch, of such problems as "metabolic models" and "metabolic pathways," of "vitamins" and "antibiotics," of "genetics of microorganisms" and "biochemical mutations," of "viruses," and of "steroid transformations," let alone such specialized subjects as "metapoeitic integrations"? By presenting a broad outline of these and other

phases of microbiology, this symposium on the *Perspectives and Horizons in Microbiology* deals with the many-sided aspects of a science devoted to the study of the microscopic forms of life and their relation to mankind.

The numerous applications of the activities of microorganisms have, for the most part, been omitted from this symposium. They are definitely suggested, however, in their relation to human and animal health, in the problems dealing with viruses and immunological reactions, in the discussion of soil processes and plant growth, and in the outline of nitrogen-fixation and the effects of microbes on plant life. The inclusion of a chapter on antibiotics tends to give emphasis to a new field of microbiology that has made great progress during the last fifteen years. The problems of disease control are suggested in the extensive applications of this advancing science.

Because of their historical interest, three general addresses delivered at the dedication of the Institute are included as an appendix.

It is the sincere hope of those who organized this symposium that it will serve as a milestone in the history of a science that deals with the smallest of living things and with their importance in the cycle of life in nature and especially in the life of man.

SELMAN A. WAKSMAN

*September 1, 1954*  
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PART **1**

THE MICROBE AS A  
LIVING SYSTEM



## Chapter **I**

# *The Microbe as a Whole*

By CORNELIS B. VAN NIEL

Until recently, microbiology was the special field of only a few individuals working in comparative isolation. This small minority regretted the tendency to equate microbiology with the study of problems in disease and public health, which commanded attention once the role of microorganisms in food spoilage and as causative agents of many plant and animal diseases had been established. These men were probably no less impressed by the great advances made in the prevention, control, and treatment of disease than were the many who had become medical microbiologists. But they realized that microbes possess a wider range of physiological and biochemical potentialities than do all other organisms combined. Microbes represent forms of life that can persist in nature because the organisms fill particular ecological niches, permitting them under special conditions to compete successfully with other living beings. Hence the few nonmedical microbiologists could envisage the unusual advantages that studies of the microbes as biological entities might offer for the attainment of a better understanding of the funda-

mental aspects of life. Occasionally they could reiterate their hope that some day the neglected opportunities would attract others to the larger field; meanwhile they continued their work, assured by a long-range point of view and by the fascination of the subject itself.

But during the last fifteen years a marked change has taken place. The number of publications concerned with microorganisms from other than medical points of view has increased enormously, and several new journals have been started to accommodate the swollen current of reports dealing with biochemical studies in which microbes have played a part. One of the chief reasons for this development has been the influence exerted by A. J. Kluyver's masterly synthesis of the information on the vast diversity of metabolic processes carried out by the numerous types of microorganisms. This synthesis revealed that all biochemical events can be interpreted as composites of more or less extended series of step reactions, each step chemically intelligible and representing a special case of simple electron transfer between two different molecules, mediated by a specific enzyme. Depending upon the nature of the participating reactants, this leads to the transfer of atoms or larger units from one molecule to another, as in transhydrogenations, transphosphorylations, transaminations, transmethyations, transacetylations, and transglucosidations.

While emphasizing the fundamental unity in the biochemical behavior of all living beings, Kluyver's contributions also drew attention to the existence among microorganisms of extreme cases of biochemical specialization and of processes that may be characterized as grossly exaggerated examples of phenomena that are encountered elsewhere as quantitatively minor side reactions. That organisms which display such properties are significant for more detailed investigations on the particular reaction mechanisms involved was clearly appreciated.

This concept gave rise to that of "comparative biochemistry," and so broadened the range of useful potentialities of microbes for biochemical studies that it is not surprising to find, in the course of fifteen years, that biochemists interested in fundamental problems became increasingly aware of the advantages offered by Leeuwenhoek's "little animals." Furthermore, the gradual realization that growth factors for yeasts and bacteria bear a close relationship to vitamins required by higher animals soon led to the use of microorganisms for the assay of vitamins and amino acids and for the detection and identification of as yet unknown growth factors. The subsequent proof that several of these substances participate in biochemical processes as co-enzymes or building blocks for coenzyme synthesis, a concept first advanced in 1933 by Lwoff in connection with studies on the role of hemoglobin in the nutrition of trypanosomes, increased the usefulness of the microbes still more, especially in view of their high rate of growth and the possibility of controlling the material by the use of pure cultures and chemically defined media. The microbes thus became the material *par excellence* for studies of special nutritional problems and of enzyme systems. And when at last methods were perfected for the extraction of enzymes from bacteria, yeasts, molds, and other microbes—through preliminary drying, disruption of the cells by grinding, shaking with glass beads, supersonic oscillations, or enzymatic dissolution of the cell walls—our understanding of the details of biochemical reaction mechanisms through the use of microorganisms advanced rapidly.

Unquestionably, biochemistry has profited greatly from these developments. Nevertheless, the microbiologist receiving a request for a pure culture of some bacterium, yeast, or alga, and for directions for growing it, from a biochemist who wishes to use it for a specific biochemical investigation cannot always escape the conclusion that the culture in question will be considered as little more than a

potential enzyme preparation. And just as the chemist will feel uneasy when a special analytical procedure or method for synthesizing a chemical compound is used by a person with little experience and no more than a rudimentary knowledge of chemistry, unaware of the rationale of the operations and of the possible pitfalls in interpretation, so the microbiologist is likely to be somewhat apprehensive when his material is treated as a chemical reagent, rather than as a mass of living organisms with their own peculiarities and responses to environmental conditions that have to be appreciated for the culture to be used to best advantage. These are aspects that can be learned, and it is comforting to know that many biochemists nowadays are anxious to acquire a sound training not only in biochemistry, but in the general principles of microbiology as well.

Even so, one might argue with considerable justification that there is now developing a strong tendency to equate "general" microbiology, as contrasted to "medical" microbiology, with biochemistry and to consider a study of microorganisms as truly significant only if it is directed towards biochemical investigations. I consider such a tendency regrettable; however, this statement should not be misconstrued to mean that I have little or no interest in biochemical problems, or that I would advocate that the use of microorganisms for biochemical studies be henceforth curtailed. Not only would such an attitude be futile; it would also be unscientific. The biochemist should not be satisfied with our present level of understanding of biochemical phenomena. He must obviously realize that many types of reactions have not yet been analyzed with respect to the nature of the enzymes involved. He must know that the interpretation of enzyme specificity on the basis of the general idea that it is related to the molecular configuration of protein molecules leaves much to be desired and that a satisfactory account of the specificity problem requires a



much more refined explanation. Even the mechanism of enzyme action through electron transfer is by no means as well understood as, for example, the reaction mechanisms between diatomic molecules. Probably, more attention gradually will be devoted to such problems, and as long as microorganisms promise to be useful for the isolation of hitherto unknown enzymes or for a more fundamental study of the mechanism of enzyme action, it would be indefensible to discourage the use of microorganisms in enzyme studies.

Nor am I concerned with the question of whether these should properly be called biochemistry or microbiology; I have little interest in definitions and delineations of fields of scientific endeavor. But I should like to emphasize that the microbe is potentially significant also for the study of phenomena that are not directly associated with either disease or enzymology, of phenomena that present problems far less clearly defined or definable but nonetheless equally fascinating. Such problems can perhaps best be indicated by referring to that realm of complexity of organization where a collection of chemical compounds exhibits the properties of a living organism. Again, I shall not attempt to define where the distinction between "living" and "nonliving" should be drawn; in the long run this may become the task for academicians desirous of formulating a useful dictionary type of definition of these terms. What I am concerned with is the attainment of a better comprehension of manifestations of matter on a level of complexity such as characterizes a microbe, implying organization, growth, and responses to environmental factors through irritability, variability, and adaptation, all of which may be combined in the term "individuality." I do not mean to express a belief that such phenomena cannot ultimately be explained on the basis of physico-chemical events. But in view of the record of the history of scientific developments, I am inclined to think that these

manifestations themselves could not be inferred from studies on isolated fragments of the organism. The mere fact that several examples have recently accumulated to show that an organism cannot use a particular substrate, although its enzymatic composition is such as to justify the conclusion that it should, is a simple case in point. At present this anomaly is attributed to permeability barriers or to enzymatic organization; it must, however, be realized that this explanation does not constitute a satisfactory one in terms of better comprehended mechanisms, but actually is hardly more than a paraphrase of the underlying observations. Let me emphasize that the point at issue is not that our current knowledge of permeability problems is still woefully incomplete, but that the mere detection of the irregularities was the result of studies with living organisms as well as with isolated enzyme extracts. Similarly, the occurrence of adaptations and mutations could not, I believe, have been surmised from experiments with crude or crystalline enzyme preparations, but had to be established by investigations with cell populations.

So far as we can perceive at present, the basis of life is matter, and the scientist must aim at an ultimate interpretation of the manifestations of life in terms of mechanisms that govern the behavior of the elementary particles of matter. To achieve this, we must know the manifestations, however, and it is my contention that we can discover them only by studies on living organisms. The greater the complexity of the latter, the more difficult will be the analysis of particular vital functions. Hence the microorganisms, in view of their relative simplicity, appear to provide promising material for further studies on the fundamental aspects of life.

Much has already been accomplished here, and there are signs of increased activity along several lines. The relatively recent developments demonstrating, for example, that bacteria in general represent organized units com-

posed of a cell wall, cytoplasmic membrane, cytoplasm, and nucleus constitute a sound basis for considering bacterial cells on a par with the cells of higher organisms. Of great promise also are the current investigations on cytological aspects, such as the discovery by Weibull that *Bacillus megaterium*, stripped of its cell wall, retains its organization inside the membrane under appropriate conditions, as well as many of its metabolic properties, although attempts to grow such incomplete cells have so far failed. In this connection a comparison with bacteria like the cytophagas, which presumably do not form a cell wall and yet can grow and divide normally, suggests itself as an interesting problem.

Similarly, the finding that the cytochromes of some bacterial cells are intimately associated with the cytoplasmic membrane, and that the pigments of the blue-green algae and photosynthetic bacteria are not, as previously believed, uniformly distributed in the cytoplasm but assembled in chromatophore-like structures, gives evidence of enzymatic organization. It may be expected that further studies of this sort will rapidly advance our knowledge.

The isolation of bacterial flagella, with the subsequent elucidation of their chemical nature as proteins of the myosin group, holds out hope for a better understanding of the mechanism that causes these "monomolecular hairs" to function as organs of locomotion. Besides, the recent work of Clayton shows that phototaxis of purple bacteria displays the typical characteristics of sensory perception, *viz.*, the all-or-none response, accommodation, and refractory period, and that the concepts and equations developed by A. V. Hill and Rashevski to account for sensory stimulation are equally satisfactory as formal expressions of phototactic behavior.

This suggests that flagellar movements are under the control of mechanisms analogous to those operative in sensory perception in general, and that further studies on

tactic movements of microorganisms might help in analyzing certain aspects of stimulus conduction by nerves.

In connection with microbial locomotion, I cannot refrain from mentioning the problem posed by the existence of *Salmonella* strains with nonfunctional flagella; the still unsolved mechanisms responsible for the movements of microorganisms devoid of flagella, like the desmids, blue-green algae, myxobacteria, and *Labyrinthula*; and the phenomena known as elasticotaxis and elasticotropism, encountered in organisms with gliding motility and in *Kurthia zopfii*, representing responses to gradients in stress of the substrate, similar to that exhibited by growing nerve cells.

It is obviously impossible to predict where future studies of such phenomena may lead. But I believe that in the end science would benefit if the tentative and often unrewarding probings into the behavior of microorganisms on a level now beyond the scope of biochemical and biophysical experimentation were not merely tolerated but encouraged. The tendency to look down on the efforts of microbiologists who do not follow the current trends, and to brand such studies as rather primitive dabbings in the natural history of microorganisms, seems to me short-sighted. It would be well to realize that the important developments in microbial biochemistry and genetics, for example, have resulted from the emergence of novel concepts that may be compared to a "break-through" in a deadlocked military front. The successful exploitation of such concepts is accomplished by the concerted efforts of many specialists, and the opportunity for doing so is generally recognized at an early stage. One cannot anticipate, however, when or where a new break-through may take place. It usually is preceded by the accumulation of numerous seemingly trivial observations which gradually arrange themselves, in the mind of an alert scientist long preoccu-

ped with a particular problem, into a pattern that initiates a new line of attack. Until this stage is reached, advance in new directions depends exclusively on individual effort. Although it is clear that our knowledge of various microbial degradations that have not yet been investigated in detail could readily be brought up to the level of understanding achieved in other cases by having teams of enzymologists, biochemists, and microbiologists work on the problems, it is futile to expect that fundamental progress in our comprehension of the mechanism of locomotion of the myxobacteria, for example, could be made as the result of team work at the present time.

Wood's discovery of the antagonistic effect of para-aminobenzoic acid on the inhibition of bacterial growth by sulfanilamide led to a new concept of the mode of action of growth inhibitors, and this opened up the fruitful field of investigations on antimetabolites. Similarly, Fleming's chance observation on the absence of bacterial colonies in the vicinity of a mold contaminant, coupled with the demonstration some years later, by Dubos and by Hoogerheide, that special substances can be isolated from cultures of spore-forming bacteria that prevent the development of other microorganisms, constituted another break-through; it could be systematically exploited by teams of specialists and marked the beginning of the antibiotics industry. The spectacular results are evident to everyone who has some knowledge of present-day medical practice.

It is important to remember that these developments have their origin in observations with mixed rather than pure cultures, and hence involve an accidental or deliberate deviation from what had become standard microbiological practice. This brings into focus the significance of studies of the behavior of microorganisms under conditions where competition among various types is not *a priori* excluded. During the last thirty years Winogradsky repeatedly insisted on the need for studying the role of

microbes in nature under such conditions, and argued convincingly that pure culture studies may reveal characteristics that can express themselves only in the absence of potential competitors. The recent discoveries concerning microbial variability and genetics also tend to discredit the once almost unlimited confidence in results obtained with pure cultures, because it is becoming increasingly evident that even populations derived from single cell isolates are anything but homogeneous. This discovery may have some disturbing implications, but it should be recognized that the appearance of variants in pure cultures can be used to study the mechanism of competition between different individuals in a clone, and thus may lead to an analysis of the factors that determine the perpetuation of specific properties. This, in turn, would provide important information for investigation in the general field of ecology.

It cannot be doubted that in the new Rutgers Institute of Microbiology the search for new antibiotics will be continued. In view of the extensive theoretical and practical background built up by Waksman during the last fifteen years, it may also be confidently expected that important advances will be made. But I hope that there will also be room for some of those who prefer to spend their time and efforts in examining properties of the microbes that have as yet eluded a more penetrating study. This will require probing into manifestations pertaining to regions of complexity which, at present, are but little understood, and may involve many kinds of observations before a significant pattern begins to emerge. Nevertheless, it is only when all the aspects of microbial behavior can be taken into account that we shall be in a position to think in terms of "the microbe as a whole."

## Chapter 2

# *Some Aspects of Metapoietic Integrations*

By ANDRÉ LWOFF

Microbes are able to undergo a variety of inheritable changes: gene mutations, loss of plasmagenes endowed with genetic continuity, transformations, or transductions. As a result, the microbe is temporarily or permanently modified. It has lost or acquired the ability to synthesize or to metabolize one or many substances. And in some cases the change has been shown to be related to the loss or the acquisition of the power to synthesize a specific enzyme.

These variations may be considered as consequences of the modification of "normal" metabolism. In some cases, however, a hereditary pathological property may be imposed on the microbe; namely, the power to produce bacteriophage or lysogeny.

As is well known, a lysogenic bacterium is endowed with the hereditary power to produce bacteriophage. This property is bound to the presence of a specific particle: the

prophage. If lysogeny were just the power to produce phage, things would be relatively simple. Instead, they are relatively complicated—though more fascinating—because the prophage not only confers on the microbe the power to manufacture phage but also may modify some of the bacterial properties. The prophage modifies the response of the microbe to infection by homologous and sometimes heterologous phages. And sometimes the prophage may endow the bacterium with properties that are, so far as we know, without any relation to phage development proper.

Before discussing metapoeitic integrations, it may be well to summarize the essentials of lysogeny. The life cycle of a temperate bacteriophage comprises three main phases: first, the infectious phase, during which the phage is in the form of the mature phage particle; second, the pathogenic, vegetative phase, during which the specific structures of the phage are produced and finally organized; third, the lysogenic phase, during which the power to produce phage is perpetuated by the lysogenic system. During these three phases the genetic material of the bacteriophage, namely, the *germ* of the phage particle, the *gonophage* of the vegetative phase, and the *prophage* of the lysogenic phase, must necessarily be the same in so far as main architecture or organization is concerned. But the germ is inert; the gonophage is multiplying rapidly, and phage proteins are produced as a result of its activity; the prophage is apparently just replicating harmoniously and synchronously with the bacterium, as if it were a normal bacterial gene.

A number of experimental data have led to the conclusion that prophage is the genetic material of the phage as modified by its attachment to a specific locus of a bacterial chromosome. In turn, the prophage modifies the properties of the bacterium as if it exerted an effect on bacterial genes. Thus the properties of a lysogenic bacterium appear



to be the result of the integration of the genetic material of the bacterium and of the bacteriophage, an integration which produces modifications and will therefore be called "metapoietic" (generating changes).

A few examples will be given to exemplify the notion of interaction of the genetic materials of the two partners of the lysogenic system. Then, some typical cases of metapoietic integrations will be described and the theoretical implications of the phenomena discussed.

### Inducibility

In some lysogenic systems the development of phage from prophage is initiated by an appropriate treatment, such as irradiation with ultraviolet light. In other systems the probability of phage production cannot be, or at least has not yet been, modified. In other words, some systems are inducible; some are not. Thus, *Bacillus megaterium* 899[1+]<sup>1</sup> is inducible, as are the lysogenized strains Mox[1+] and M[1+]; on the other hand, *B. megaterium* 17[2] and the lysogenized strains Mox[2] and M[2] are not inducible.

In the double lysogenic *B. megaterium* 17[2], [1]—inducing agents elicit the development of phage 1 only. It was therefore concluded (6) that inducibility was a genetic property of the prophage, a conclusion which, as will be seen, is at the same time true and untrue.

In the course of the study of *B. megaterium*, a defective inducible strain, *B. megaterium* 91[1+], was obtained by lysogenization. The bacteria of this strain undergo lysis after irradiation with ultraviolet light but do not produce bacteriophage. The defective strain retained its inducibility for two years. During the third year inducibility disappeared. Moreover, instead of producing the original phage 1+, this strain now produced two phages, GC (G

<sup>1</sup> The number in brackets is the identification number of the prophage.

for great, and C for clear) and 1GT (T for turbid), both being temperate. And it turned out (see Table I) that lysogenic strains M[1GT] and Mox[1GT] were inducible, whereas M[1GC] and Mox[1GC] were not. Then, from the noninducible, defective lysogenic strain, a nonlysogenic variant was isolated which will be called 91B. This strain, when lysogenized with phages 1+, 1GT, or 1PT (P for *petite*), gave rise to noninducible systems only, whereas the strains M and Mox, lysogenized with the same phages, gave rise to inducible systems.

**TABLE I. Inducibility in *Bacillus megaterium* as Controlled by the Genetic Constitution of the Bacterium and of the Bacteriophage \***

Inducible Systems	Noninducible Systems
899[1+] M[1+] Mox[1+] 17[2] [1+] 10[3] [1+] M[1GT] Mox[1GT] M[1PT] Mox[1PT]	91 B[1+]     91 B[1GT]  91 B[1PT]  M[1GC] Mox[1GC] 91 B[1GC]
10[3] M[3] Mox[3] 91 B[3]	

\* From the results of Ionesco (5, 6).

Thus a given phage may produce an inducible system in a given bacterial strain and a noninducible system in another.

When strains Mox and M were lysogenized with the phage produced by any of the noninducible lysogenic strains 91B[1+], 91B[1PT], 91B[1GT], only inducible

systems were obtained. In so far as inducibility is concerned, therefore, the phages produced by the lysogenic noninducible strains 91B give rise to inducible systems in the appropriate bacterial strains M and Mox.

Among the phages of *B. megaterium* is phage 3, which is inducible in the original strain *B. megaterium* 10[3], as well as in the lysogenized strains M and Mox. Moreover, strain 91B[3] is also inducible. Thus, the development of phages 1 and 3 is inducible in the lysogenic systems M and Mox, whereas in the lysogenic 91B, only the development of phage 3 is inducible.

Inducibility is apparently a genetic property of neither the phage nor the bacterium but a property of the lysogenic system as a whole. We have no right to speak of inducible or noninducible prophage. It is the lysogenic system which is or is not inducible, and inducibility is obviously the result of the interaction of the genetic material of the phage and of the bacterium.

### Defective Strains

In a "normal" inducible lysogenic strain, virtually all the bacteria will produce phage after irradiation with an adequate dose of ultraviolet light. In a "defective" strain, only a small fraction of the bacteria—of the order of  $10^{-5}$ —will produce phage after irradiation, despite the fact that phage development has been initiated by the inducing agent. All bacteria, nevertheless, die as a consequence of an abortive development of phage.

The defective lysogenic *B. megaterium* 91[1+] may lose its prophage. The nonlysogenic strains thus obtained, when lysogenized, prove to be normal. Moreover, the phage produced by the original defective 91[1+], when infecting a nonlysogenic bacterium, gives rise to normal lysogenic systems.

It seems as though neither the genetic constitution of

the phage nor that of the bacterium is responsible for the defectivity. The most likely hypothesis is that defectivity is correlated with an abnormal anchorage of the genetic material of the phage on the bacterial chromosome. Defectivity is frequently observed in *Pseudomonas pyocyanea*. Let us infect an apparently homogenous population of bacteria with an apparently homogenous population of bacteriophage and isolate lysogenic clones. The fraction of bacteria able to produce phage varies with the clones from 1 to  $10^{-4}$ . Here also, the defective as well as the normal lysogenics produce the same type of phage. And again, the simplest hypothesis is that normality or defectivity is the consequence of the mode of attachment of the genetic material of the phage to the bacterial chromosome. It would, nevertheless, be dangerous to generalize from this hypothetical conclusion. The lysogenic *B. megaterium* 10[3] is defective. The strains M and Mox, when lysogenized with phage 3, give rise exclusively to defective strains. Here defectivity is controlled by the genetic constitution of the phage. The most general expression of these hypotheses would be that normality and defectivity are controlled by the genetic constitution of the phage as modified in its expression by bacterial genes. Here again, the character normality-defectivity is, in the last analysis, controlled by the genetic constitution of the system as a whole.

### *Immunity and Dysgonia*

We have considered so far such properties of the lysogenic systems as are connected with the development and maturation of bacteriophage from prophage. It is well known, however, that prophage not only confers on the bacterium the power to produce bacteriophage, but also endows it with a specific immunity. The "homologous" phage, the phage corresponding to the prophage, as well

as some of its mutants, can be adsorbed on the lysogenic bacterium. The genetic material of the infecting phage passes into the bacterium, but it neither enters the vegetative phase nor is converted into prophage. It is not reproduced. So far as we know, it is not pathogenic. It is diluted at each bacterial division and behaves as an inert protoplasmic particle. This is immunity.

If the prophage develops in an immune superinfected lysogenic bacterium, then the genetic material of the superinfecting phage will also enter the vegetative phase. Immunity, which corresponds to a block in the development of the superinfecting phage, disappears when the prophage, entering the vegetative phase of the cycle, ceases to be a prophage. Immunity is apparently bound to the prophage as such.

The two fundamental properties of the lysogenic system, the hereditary power to produce bacteriophage and immunity, are specifically controlled by the specific prophage and may be considered—somewhat arbitrarily perhaps—as representative of its essence. With the study of dysgonia we enter the field of nonspecific effects of the prophage.

Many cases are known in which the presence of the prophage modifies the response of the lysogenic bacterium to "heterologous" phage, that is to say, phages nonserologically related to the prophage. The modified response of the lysogenic system may be related to phage development or to lysogenization. Let us consider *P. pyocyanea* and phages 4 and 8, which are not related serologically. The nonlysogenic *Pseudomonas* infected with phage 8 gives rise to phage with a probability of one. If a lysogenic bacterium-perpetuating prophage 4 is infected instead, the following results are obtained: 2 to 5 per cent of the infected lysogenic bacteria produce phage 8, 50 to 60 per cent die without producing any phage, 40 per cent survive. Thus the response of *Pseudomonas* to phage 8 is modified by the presence of prophage 4.

Both bacteriophages 4 and 8 are temperate. But the presence of a given prophage can also prevent the development of a virulent phage. Thus the lysogenic *Shigella dysenteriae* perpetuating the prophage  $P_1$  does not allow the development of the phages  $T_1$ ,  $T_3$ , and  $T_7$ , whereas it allows the development of  $T_2$ ,  $T_4$ ,  $T_5$ , and  $T_6$  (2). The situation is the reverse for the lysogenic  $P_2$ , which allows only the development of  $T_2$ ,  $T_4$ ,  $T_5$ , and  $T_6$ . None of the T phages is able to mature in the double lysogenic  $P_1$ - $P_2$ . This block in the reproduction of T phages is suppressed by the action of various inhibitors of the metabolism (7). It is, therefore, due not to the prophage as such, but to an alteration of the bacterial metabolism brought about by the presence of prophage.

It is interesting to know that *Escherichia coli* B[ $P_2$ ] allows the development of phage  $T_2$ ,  $T_4$ ,  $T_5$ , and  $T_6$  (7). Thus the prophage  $P_2$  does not exert the same effect on the bacterial metabolism in *E. coli* as in *S. dysenteriae*. Here again, it seems clear that the properties of a lysogenic system are not "produced" by the prophage, but are the result of the interaction of the genetic material of the prophage and of the bacterium. The nonspecific action of a given prophage depends on the genetic constitution of the bacterium.

Another example of dysgonia has recently been discovered (1). *E. coli* K12 allows the development of phage  $T_{2r}$ , whereas the infection of *E. coli* K12( $\lambda$ ) is not followed by phage production. Moreover, phage  $T_{2r}$  does not develop in the lysogenic bacteria in which the development of phage  $\lambda$  has been induced by irradiation with ultraviolet light. This is true even if the infection is performed 40 minutes after irradiation, that is to say, after the onset of the vegetative phase of phage  $\lambda$ . The conclusion is that the altered response of the lysogenic bacteria is due not to the prophage as such, but to a modified state of the bacterium brought about by the prophage.

The presence of a given prophage may also block the

process of lysogenization. *S. dysenteriae* [ $P_2$ ] can be lysogenized by phage  $P_1$ , and double lysogenics are thus obtained. When phage  $P_2$  is added to lysogenic *S. dysenteriae* [ $P_1$ ], phage  $P_2$  is adsorbed, but it neither develops nor is converted into prophage. A lysogenic *S. dysenteriae* [ $P_1$ ] infected with phage  $P_2$  survives and remains lysogenic  $P_1$ . Thus the presence of prophage  $P_1$  blocks the development of  $P_2$  and its conversion into prophage (2).

### Alterations of Bacterial Metabolism

A lysogenic bacterium is modified in its ability to reproduce the homologous phages, to reproduce some heterologous phages, and to convert a phage into prophage. The responses of a lysogenic bacterium to phages are modified. In this last section we shall discuss cases in which the presence of prophage brings about modification of the bacterial metabolism and which are apparently not related to any known event in the life cycle of bacteriophage.

The first and classical example is the synthesis of a specific toxin by *Corynebacterium diphtheriae*. Only lysogenic strains carrying prophage  $\beta$  are toxinogenic, and non-toxinogenic strains, when lysogenized with phage  $\beta$  become toxinogenic (3). When the prophage  $\beta$  is suppressed in a lysogenic-toxinogenic strain, the toxinogenic power is lost (4). Bacteria lysogenized with any phage  $\gamma$  are not toxinogenic. Perhaps phages  $\beta$  and  $\gamma$  are related, but whatever the case may be, there is no cross immunity between them. The interesting point is that lysogenic types may be obtained which exhibit a combination of properties of lysogenics  $\beta$  and  $\gamma$ ; namely, immunity to  $\gamma$ , as in the lysogenic  $\gamma$ , and toxinogeny, as in lysogenic  $\beta$  (type 2 of Table II). Things happen as if a recombination had taken place between the genetic material of phage  $\beta$  and  $\gamma$  (4). Apparently, toxinogeny is controlled not by prophage  $\beta$  as a whole but by a specific part of its genetic material,

TABLE II. Control of Toxinogeny in *Corynebacterium diphtheriae* \*

	Immunity to $\beta$	Immunity to $\gamma$	Toxinogeny
<i>C. diphtheriae</i>	0	0	0
<i>C. diphtheriae</i> [ $\beta$ ]	+	0	+
<i>C. diphtheriae</i> [ $\gamma$ ]	0	+	0
<i>C. diphtheriae</i> [ $\gamma$ ] [ $\beta$ ] †	+	+	+
<i>C. diphtheriae</i> [ $\beta$ . $\gamma$ ] type 1	+	0	0
<i>C. diphtheriae</i> [ $\beta$ . $\gamma$ ] type 2	0	+	+

\* From the results of Groman (4).

† The status of this strain is not yet clear. Types 1 and 2 have been isolated after exposure of *C. diphtheriae* ( $\gamma$ ) to phage  $\beta$ .

which may be associated with a structure conferring immunity against phage  $\gamma$ .

Here, the presence of a prophage of a given genetic constitution modifies the bacterium in such a way that it produces toxin when deprived of iron.

In other cases, the presence of a prophage modifies the metabolism of the microbe so that the morphology of the colony is altered. In *B. megaterium*, the colony type is changed by lysogenization with phages IPT and 3, not with phages IGC and 2. The morphology reverts to the normal when the prophage is lost (5). In this case, as in the case of *Corynebacterium diphtheriae*, the hypothesis of a selection by the phage of a preexisting bacterial mutant has been ruled out. The new bacterial feature is due to the presence of prophage and disappears when the prophage disappears.

## Discussion

A cell is an organized totality. The importance of the interrelations and interactions of cell particles and organelles has long been recognized, and the life and reproduction of a cell are often visualized as the result of integrations at various levels of organization.



Obviously, the properties of lysogenic systems which are bound to the prophage are the result of interaction of the genetic material of the phage and of the bacterium. Many hypotheses can be considered to account for the alterations of the bacterial metabolism that are the result of lysogenization. It could be that the prophage modifies the bacterial genes adjacent to the locus of attachment. It could also be that some of the prophage genes actively intervene in a specific process, such as the synthesis of a protein or of a part of a protein. We are unfortunately, for the time being, left with hypotheses. Whatever the solution—or solutions—of the problem may be, it is possible to conclude that the genetic material of the bacteriophage, when attached as prophage to a bacterial chromosome, is not solely a potential bacteriophage. The prophage takes part in the daily life of the bacterium as if it were a cog in the bacterial machinery. It plays its part in the molecular orchestra.

Thus, the structural basis of a viral disease and the bacterium may be integrated. Some of these integrations have been recognized as metapoietic.

It is certain that many more examples of such integration will be brought to light. The mechanism of the phenomenon will have to be disclosed.

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## Chapter 3

# *Genetics and Microbiology*<sup>1</sup>

By JOSHUA LEDERBERG

“To the biologist of the 19th century, bacteria appeared as the most primitive expression of cellular organization, the very limit of life. In reality it appears that it is only their small size and the absence of recognized sexual reproduction which has given the illusion that bacteria are simple cells.” Thus Dubos (11) introduced an outstanding synthesis of the structural and biochemical complexity of the microbe as a living system. But in another realm, he (12) could not repress a nostalgia for primitive simplicity: “Bacterial variation passes from the collector’s box of the naturalist to the sophisticated atmosphere of the biochemical laboratory. One may wonder whether the geneticist will not arrive too late to introduce his jargon into bacteriology.”

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Alas, it could not be helped; the same symposium (Cold Spring Harbor, 1946) was already teeming with cytogenes, mutagenesis, allelomorphs, recombination, and heterozygotes, and microbiology had already succumbed.

To an impatient bystander this fusion—or confusion—of disciplines might seem overdue. Mendel was, after all, a contemporary of Pasteur, and could we not imagine their communication and understanding that the abbot's "Anlagen" were the stuff of unspontaneous generation? But Pasteur was a prophet honored in his own time, while Mendel was first ignored and then forgotten, and the burgeoning science of microbiology grew up without benefit of eugenic supervision.

As a science, genetics dates from the exhumation of Mendelian analysis at the turn of the century; as a term, it was first coined in 1906. Genetics might have been infused with microbes from the start, with Blakeslee's discovery of the segregation of fungal sex factors in 1904, had the zygospores of the mucors germinated more readily, but he turned to more amenable plants for his genetic work, and he works with them still.

Heredity has always been an object of avid curiosity and inquiry. Darwin, for example, was obliged to consider some mechanism of inheritance to underlie his evolutionary theory and, with some misgivings, adopted a Lamarckian concept whereby the progeny resemble their parents whether for innate germinal, or incidental somatic peculiarities. This fallacy was eroded by Weissmann and (for higher organisms) laid to rest in the 1900's with the Johannsen and de Vries delineation of pure lines and their mutations. Prior to Mendelism, strong circumstantial evidence already supported widespread belief that the chromosomes were the primary vehicle of heredity.

Meanwhile, the bacteria were and remain a convenient repository for hypothetical evolutionary starting points and speculated genetic mechanisms that might be refuted

by Mendelian and populational analysis elsewhere. To be sure, such pioneers as Massini, Beijerinck, and Barber did distinguish fluctuating or impressed variations, which are reversible physiological responses to the environment, from fixed mutations representing innate genetic alterations. Bacterial mutations are reversible, however, in the same sporadic fashion as the primary events; reversion is no less characteristic of other organisms, but this was overlooked in support of cyclogenic or more obscure special theories of bacterial dissociation (18). The confusion was compounded by the tremendous size of bacterial populations and the aggressiveness with which bacterial mutations and reverse mutations often present themselves (6, 33).

These features were put to good use in a renewal of exact study by a biometric approach in the 1940's, culminating in the analysis of clonal variance of mutations to phage resistance by Luria and Delbrück (34). The outstanding qualitative result of these experiments was statistical proof of the uncontrollability, if not the absence, of purposive regulation of adaptive mutations, such as resistance to phage or to streptomycin. The statistical procedures have since been expanded for the calculation of mutation rates and for more detailed analysis of spontaneous mutation (2, 38). But population dynamics is so complex (7) that foreseeable progress here may consist of balancing the complications omitted from the approximate calculations against the imprecisions of measurement. The clonal variance that is the keystone of the qualitative argument for preadaptive mutation also requires tedious repetition to measure mutation rates with a scarcely acceptable precision of  $\pm 50$  per cent (34, 38, 42). Ordinary cultural methods also entail an ever-changing chemical environment (7) in addition to an inexhaustible reservoir of systematic as well as sampling errors (26).

Novick and Szilard (39) have resolved these obstacles by means of a simply engineered continuous-flow device,

the chemostat, in which cultures can be maintained in a self-regulated, steady-state environment long enough to submerge short-term random fluctuations. Accordingly, spontaneous mutations can be measured with a precision of 5 to 10 per cent and an assurance that nonlinear perturbations of the steady state are either self-cancelling or so exaggerated as to be self-evident. Sophisticated studies of bacterial mutation can no longer afford to ignore the experimental control uniquely offered by this approach.

“Experimental control of spontaneous mutation” is an intentional paradox. “Spontaneous,” despite purposeful misreading by dialectic materialists, means neither the immateriality of the gene nor the notion that genetic material lacks a physical nexus with the environment (19). It does mean that the subtle chemistry of the gene has evolved through conservative mechanisms so refined that we cannot discern their connection with the end products of organic structure and function; at least we have not yet learned how to distinguish one gene from another by reactants that allow purposeful, specific changes. Some day the secret of specific mutagenesis will be revealed, but such faltering claims, for example, as that antibodies might alter specific genes have not held up (42). To my mind, the search for this philosopher’s stone by bludgeoning the bacterial gene with drugs or enzyme inhibitors will some day seem as credulous as the snipping of mouse tails does now, and it has already had equally negative, if sometimes glittering, results. [I cannot overlook the remarkable effects of one inhibitor, acriflavine, on respiratory factors in yeast (13), but this seemingly embarrassing exception has illuminated the path from infection to heredity (9, 28)—the effect is akin to the “chemotherapeutic cure” by streptomycin of green plants with regard to their chloroplasts (41, 48).]

Experiments with the chemostat have demonstrated significant environmental control over rates of sporadic muta-

tion of various bacterial genes. Not only a rise in temperature, but also such metabolic variables as tryptophane deficiency or adenine excess accentuate the mutation rate (40). Perhaps the most provocative finding was that ribonucleosides would not merely reverse the effect of excess adenine but would reduce the "spontaneous" rate by half (39). Biophysicists once speculated, and then rejected, cosmic radiation; we now observe that the causation of spontaneous mutation is a problem incidental to intermediary metabolism.

This conclusion converges with the explicit study, largely with microorganisms, of mutations induced by ionizing radiations and chemical reagents. "Induced" may be as misleading as "spontaneous"; it implies contrived increase of the over-all mutation rate, in a sense, an augmentation of "spontaneous" change. Between 1928 and 1940, the only accepted mutagen was radiant energy, many chemicals having been inconclusively or at least unconvincingly tested (3, 36). But starting with the war gases, and now including such domestic articles as hydrogen peroxide, formaldehyde, soporific drugs, and caffeine, so extensive a catalog has been shown potent (3, 10) that one wonders what, besides rigidity of concept, can have hindered the germination of chemical mutagenesis for so long. Now, from a free interchange of results concerning microorganisms and macroorganisms, ionizing radiations also are inferred to work through chemical intermediaries: free radicals from oxygen and water (45). And, first with *Streptomyces* (22), then with other organisms, the dysgenic effects of ultraviolet have been found reversible by visible light, suggesting an obscure photochemical intermediation.

Mutagens have, of course, helped to furnish variants, the raw material for other experiments. But, particularly in industrial applications, there has been exaggerated emphasis on the technology of provoking genetic variation (which

is relentless anyhow) at the expense of thoughtful search for procedures to detect and electively enrich the variants that serve some specific purpose, an approach we have inherited in large part from the Delft school of microbiology. Some helpful methods have been developed (32) for the isolation of the biochemical variants that Davis has so skillfully exploited.

### ***Genetic Recombination***

All this bears more heavily on the general utility of microorganisms in genetic research than on the genetics of particular microbes. Once given that microbes are differentiated into more than the rhetorical "bag of enzymes," as already proved by mutation research, we must look more deeply into the organization of different microbes, and for this, *genetic recombination* is the most versatile tool now at hand. After all, most experimentation consists of putting together two reactants and waiting to see what happens. Synthetic chemical reagents are too crudely designed for us to tell very much about their ultimate effect on genetic configuration, and we therefore seek the means of combining the biological units themselves. Later, if we can keep busy, patient, careful, and lucky, we may hope to build genetic theories on cytological facts, but the present foundation of unarguable correlations is still unsteady, perhaps owing to technical difficulties as much as to the unavoidable subjectivity of cytological conviction. If they are cells, bacteria do have organized nuclei and some approximation to mitosis, but we lack the very criteria of proof on which we can readily judge current controversies on particular manifestations of these forms.

Genetic recombination includes any process of the coalescence within one cell or organism of genetic factors from two or more parents. Its best known manifestation

is sexual fertilization: a union of an entire gamete nucleus from each parent to form the hybrid, zygote nucleus. There are, of course, innumerable secondary physical and psychic paraphernalia to support the act of fusion, but this and not the accessories is the essence of sex for genetic purposes. Sex occurs universally among higher plants and animals and is nearly as prevalent among protozoa and fungi, but, with sporadic exceptions, has been reported absent from the bacteria.

Among lower plants, we also discern another mode of recombination—heterokaryosis—in which the interaction of intact nuclei falls short of fusion. Instead, diverse nuclei multiply *sui generis* in a common cytoplasmic pool, where their functional contributions are so intermingled as to simulate hybridity. By sporulation, accident, or surgery, however, the nuclei of the heterokaryon may be segregated to reveal their lasting integrity. In the higher fungi, a binary heterokaryon is a regular feature of the life cycle, is maintained by conjugate mitosis, and may be ultimately terminated by sexual fusion, whereas in the ascomycetes and the phycmycetes, the nuclei of heterokaryons multiply independently. A similar type of heterokaryosis almost certainly occurs in actinomycetes (27); among nonfilamentous bacteria it could only be transitory, but cannot be ignored in the momentary control of phenotypes in mutation and recombination procedures.

By 1945, morphological approaches to the question of bacterial sexuality had raised so many vexatious controversies that, to quote Dubos' book (11) again: "If bacteria do really reproduce by sexual methods, it should be possible to cross closely related species and strains . . . most workers who have attempted to cross related strains have reported only failure."

Hindsight suggests that the chief omission of previous experiments (14, 43) was a set of clear-cut, unit markers in a selective system that would allow the detection of in-



frequent recombinants. But as early as 1908, Browning (8) did, in fact, make an impeccably designed test of sexuality in trypanosomes, using drug resistance for selective markers. In 1946, this forgotten experiment was repeated with some strains of *Escherichia coli*, and has since provided grist for the mills of several laboratories (30, 46). The main conclusions of the recombination analysis point to the participation of intact cells in a sexual interaction, and it is called sexual because the putative gametes encompass the whole genetic content of each parent (37). Selective methods were originally necessary because of the infrequency of recombination, which also precluded a parallel morphological decision.

More recently, especially favorable strains with higher fertility have facilitated microscopic studies, and in appropriate mixtures of cells conjoined pairs have been seen and isolated with the micromanipulator (27). If left undisturbed, the swimming pairs will disjoin in an hour or two. Exconjugants from about half the pairs engender detected recombinants (and other sexual progeny are doubtless undetected with the particular markers used). The recombinants issue from only one, the maternal parent, so to speak, to imply that conjugation transfers a nucleus from one cell to the other, followed by fertilization and reduction, rather like either half of a paramecium mating. No distinctive zygote structures, aside from the pairs, have been noted. Except that it submits to considerable stretching and torsion, nothing has been seen directly of the conjugal apparatus, probably because of optical limitations. A good deal remains to be learned, but I want to emphasize that this experiment is validated primarily by the genetic, not the morphological, observations.

Although about 5 per cent of *E. coli* strains are known to be fertile, sexual recombination has not been verified, or at least studied genetically in any recorded detail, in other bacteria, though a number of leads are being in-

vestigated in several places. Genetic exploration of various morphological representations of bacterial sex is overdue.

### *Genetic Transduction*

We now depart from mechanisms that should be familiar to every student of general biology, and must deal with a unit not previously recognized in genetics: the hereditary fragment that defines *genetic transduction*. In 1928, Griffith transformed the capsular specificity of rough pneumococci by heat-killed vaccines of smooth types (16). One is again forced to acknowledge the fortuitous success enjoyed by some irrationally designed but well-executed experiments. Although not cited by Griffith, the literature of the preceding decade (26) carried many accounts of paragglutination, whereby a superficial attachment of heterologous antigens to bacterial cells was misconstrued as hereditary alteration. Griffith, however, soon realized (as some of his successors have not) that the transformation could not concern merely the capsular polysaccharide, but must involve the machinery for its formation, as we would now say, a "genetic" or metapoietic factor. Griffith also conceded the theoretical qualification that his vaccines contained residual bacteria not revealed by conventional sterility controls, but resuscitated in the experimental mixtures. This caution has often been overlooked and can be disposed of only with the help of strains differentiated by several markers (23, 24), as was eventually done with the pneumococcus also (4, 20).

Unfortunately, the occasional notice taken of Griffith's work by genetically minded workers was often confused by the prescription of "directed mutation" and perhaps by the indiscriminate use of "transformation" for any species of change; it was not for another twenty years that this transformation was again generally accepted (36) as

an example of fragmentary genetic transfer, that is, *transduction*. Meanwhile, the chemical analyses by Avery and his colleagues described the reagent in the vaccines as principally, if not exclusively, desoxyribonucleic acid, DNA (4). The genetic aspects of the transmitted fragment have not been fully clarified, but in each of the several examples of transduction, single genetic factors are the rule, punctuated by occasional "linked transduction" of two factors (20, 31, 44). As the number of factors examined increases, so does the incidence of recognized linkages. This suggests that the unit is a chromosome fragment, rather than an absolutely delimited macromolecule, the idealized "single gene."

Transduction in at least two other bacteria, *Hemophilus* and *Neisseria*, was discovered by conscious emulation of the Griffith and Avery procedures, and with some advantageous peculiarities, its general aspects are the same as in the pneumococcus (31). In *Salmonella*, on the other hand, transduction was accidentally discovered in the course of a fruitless search for sexuality as it occurs in *E. coli*. In fact, too rigid insistence on the use of double mutants to control the selection of recombinants nearly obscured the initial discovery (49), but this emphasizes the difference in mechanism. Transduction in *Salmonella* differs from that in the pneumococcus primarily in the function of temperate bacteriophage as the passive vector of the hereditary fragments.

To recapitulate, genetic transduction as much as sexual fertilization is an agency of recombination but differs in two principal features: morphologically, one reactant is a subcellular fragment, and genetically, perhaps as a corollary, a small fragment rather than the entire genotype is all that is transmitted. In several bacteria, the fragment may be transmissible after chemical extraction in essentially native form, possibly pure DNA, but in *Salmonella* a symbiotic phage effects the initial fragmentation of the

bacterial nucleus, the intercellular transport of the fragment, and its injection into the new host bacterium.

Insofar as we elect to regard hereditary viruses as part of the genotype, symbiotic infection is also a species of recombination. Lysogenization in particular can be analogized to transduction or even fertilization (15), especially since, at least in *E. coli* K-12, the prophage is incorporated as if it were a typical genetic factor (25). In addition, traits usually attributed to the "bacterium itself," whatever this means, may be dramatically converted by lysogenization *per se*, as in the toxigenic variation of diphtheria bacilli (5, 17) and the change in lysotype (1) or somatic antigen from group E-1 to E-2 (21, 47) in *Salmonella*.

It is less urgent to distinguish whether lysogeny is a transduction, which is mostly semantic manipulation, than to describe the role of the phage particle. This may be considered as a miniature bacterium, with a skin and a "nucleus." The phage nucleus itself is the agent of genetic conversion in lysogeny; it behaves rather as if it were a special segment of a bacterial chromosome, but as with any virus we cannot say whether this evolved by gradual parasitic degeneration or by abrupt mutation.

In addition to the phage nucleus, the skin may enclose other fragments, the residue of the lysed bacterium. The relationship of these fragments to the prophage in *Salmonella* is obscure. The simplest picture is that they are adventitiously included, together with the phage nucleus, during the maturation of the phage particle. However, it has not yet been possible to study the localization of prophage in *Salmonella* by the methods employed for *E. coli* K-12, and it cannot, therefore, be excluded that the fragments transduced by any given phage particle are related to a less rigidly predetermined reproductive site of the nucleus of that particle. In any event, every genetic factor so far tested in *Salmonella* is subject to transduction, although the quantitative efficiency may vary by as much as

fiftyfold from one factor to another. Another phage-mediated transduction has been found in *E. coli*, but this is rigidly limited to a cluster of factors (for galactose fermentation) closely linked to the prophage site (25, 35), and a special relationship of the transduced fragment to the developing phage is therefore certain.

The distinctive features of phage-mediated transduction in this context are: (a) the transductive competence of any crop of phage is determined entirely by the genotype of the host cells from which the crop is obtained, and (b) lysogeny is separable from the transformation, that is, transduction may be consummated without the necessary establishment and maintenance of the lysogenic state, and recipient bacteria may be lysogenized without usually manifesting other genetic changes. In the lysogenic conversions, the competence of the phage is essentially independent of the host, and lysogeny is both necessary and sufficient for the concomitant changes. It is conceivable that these conversions are a relic of "bacterial" genes not yet redifferentiated in the phylogeny of the phage, but the chief virtue of such ethereal speculations is to emphasize the ambiguity of our concepts of organismic individuality (8).

### ***Potentialities of Recombination Methods***

Why emphasize the prospects of recombination over other means of genetic analysis? First of all, it should lead to the substantiation of life cycles (compare alteration of generations in plants and animals), but we must confess that the L-forms have eluded genetic analysis. Then, recombination is indispensable for understanding other modes of genetic variation. For example, it furnishes proof that the effects of acriflavine in yeast, already noted, are cytoplasmic depletions rather than directed gene mutations, while in lysogenic *E. coli* it has fixed the genic locali-

zation of the prophage, which had been thought a likely inhabitant of the cytoplasm.

Recombination also gives logistic support to other experimentation, for example, in biochemistry or immunology, by allowing the rational construction of prespecified combinations of genetic factors. The potential of recombination methods in applied microbiology should be so obvious as to obviate comment but has generally been overlooked in favor of more routine screening methods.

Finally, but not exhaustively, the very occurrence of recombination illuminates both taxonomy and evolution, for example, in rationalizing the otherwise unintelligible list of serological types of *Salmonella* (29). These findings, in turn, may reopen the question of how much reliance should be placed on serological typing of *Salmonella* as a clinical rather than an epidemiological tool. Although distinctions between serotypes are entrenched in the public health laws of many states, supposedly innocuous serotypes such as *S. typhimurium* are too often ignorant of the legal definitions of paratyphoid fever.

To offer seriously any prescription for the future of science would require a calculated blend of presumption and inattention to history. If such predictions were accurate, we would be disappointed, for they are the measure of the bounds of current thought that we hope to overreach. The perspectives of current microbiological science might be mistaken for prophecy; but the wisest prophet would look beyond the visible horizon for the questions we are not yet ready to ask.

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## Chapter 4

# *Nutritional and Enzymatic Studies on Microbial Mutants*

By BERNARD D. DAVIS

Some fifteen years ago, Beadle and Tatum initiated the systematic investigation of auxotrophic mutants of a microorganism. Nutritional studies on such mutants indicated that each was blocked in a single biosynthetic reaction, presumably because it had lost the ability to synthesize the enzyme that catalyzed the reaction. On the genetic side, these results gave rise to the famous one-gene-one-enzyme hypothesis, which proposed that each gene provides information that determines the structural specificity of a corresponding enzyme. On the biochemical side, auxotrophic mutants of molds and also, more recently, of bacteria have been studied in an increasing number of laboratories, and this work has led to the detection of a large number of intermediates in the biosynthesis of various amino acids, nucleic acid bases, and vitamins.

One criticism of the one-gene-one-enzyme hypothesis has been that it was built on enzymatic interpretations that,

in turn, were based only on indirect, nutritional evidence. I should therefore like to describe the results of enzymatic studies that my colleagues and I have undertaken on extracts of a variety of mutants. Furthermore, I should like to consider in some detail the biochemical significance of these findings, for they permit us to judge, much more rigorously than before, whether compounds revealed by nutritional and accumulation studies are true, normal metabolic intermediates.<sup>1</sup> Finally, I shall point out how

<sup>1</sup> I should like to propose the following definitions:

An *obligatory intermediate* is a member of a path that is the only one by which an organism can synthesize a given product at an adequate rate from given source materials. Most of the known biosynthetic paths belong to this category, since a block in almost any one of them gives rise to a growth requirement.

It should be emphasized that obligatory intermediates must be defined with respect to the beginning as well as the end of the metabolic path under consideration. Thus, in various organisms growing on glucose and ammonia alone, it is well known that cystathionine and homocysteine are obligatory precursors of methionine. Replacement of the glucose by other general carbon sources, such as other carbohydrates, acetate, or glycerol, would not be expected to change this path. Addition of the  $\alpha$ -keto acid corresponding to methionine, however, could produce such a change; for though this compound is not on the path between carbohydrates and methionine, it can be nonspecifically transaminated by some organisms to form methionine. In consequence, cystathionine and homocysteine would no longer be obligatory intermediates when an organism that could transaminate this keto acid at a sufficient rate was grown in a medium containing an excess of the compound. In some analogous cases, it has been demonstrated by the method of isotopic competition that the presence of certain precursors of amino acids can completely eliminate the formation of those amino acids from general carbon sources and hence completely eliminate the participation of the earlier intermediates (1).

We shall consider an *essential intermediate* to be one that is obligatory on a *minimal medium* (that is, a medium containing only general carbon sources, any growth factors required by the wild type, and inorganic salts).

If the term *normal intermediate* is to have any useful meaning, it seems necessary to restrict its application to the use of source materials that are defined as normal; otherwise all metabolites produced by an organism would be normal, and all those that could be incorporated into some metabolic product would be normal intermediates. I would suggest that a normal medium be considered synonymous with the minimal medium. Then on a biosynthetic path for which there is no alternative, a normal intermediate would be synonymous with an essential intermediate. Where there are minor alternative paths (as in the formation of valine by

a fusion of enzymatic and nutritional studies on mutants has made it possible to solve problems that could not be solved by either approach alone.

Since several of the enzymatic studies to be described have been concerned with a path of aromatic biosynthesis, I shall first present this path in some detail.

Our interest in aromatic biosynthesis arose through the chance isolation of several bacterial mutants that required a mixture of four aromatic compounds: tyrosine, phenylalanine, tryptophan, and para-aminobenzoic acid (9). Subsequent work showed that most of these strains had a relative requirement for traces of a fifth aromatic compound not previously known to be a bacterial metabolite, para-hydroxybenzoic acid (8). Since then, we have found that under certain circumstances these strains require in addition traces of a sixth factor, as yet unidentified (Figure 1).

The common aromatic structure of these several compounds led to a search for a possible common precursor. It was found that a rare plant acid, shikimic acid, could satisfy the growth requirement of several of these aromatic polyauxotrophs. Furthermore, the response was proportional to shikimic acid concentration and was approximately the same as that produced by a molar equivalent of the required mixed aromatic supplement. Finally, the other strains with the same requirement, presumably blocked in a later reaction in the same sequence, could not respond to shikimic acid, but could be shown to accumulate large quantities of this substance in their culture filtrates. We therefore felt justified in concluding that shikimic acid is a precursor of these aromatic cell constituents (4).

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transamination of  $\alpha$ -ketoisovaleric acid with alanine rather than with glutamic acid (27)), normal intermediates would include both essential and non-essential ones; where there are major alternatives (as may be true in carbohydrate metabolism preceding specific biosynthetic paths (2)), none of the normal metabolites would be essential.

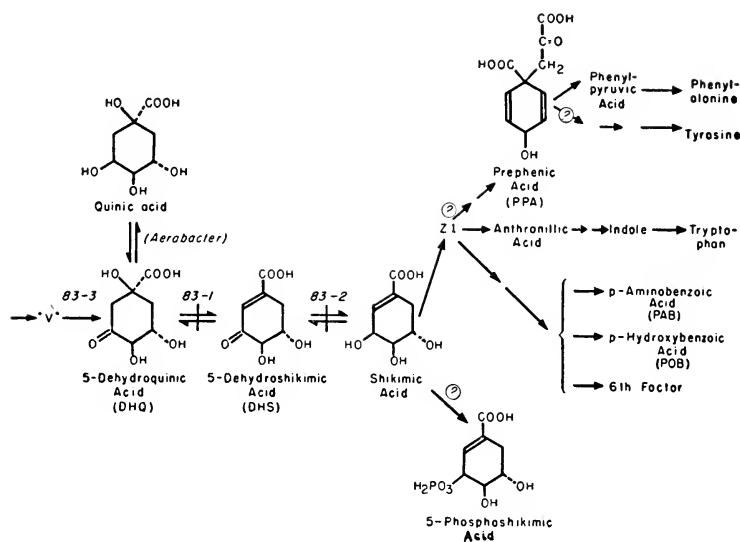


FIGURE 1. Path of Aromatic Biosynthesis

Mutants blocked immediately before shikimic acid accumulated a precursor of this compound that was readily recognized as a growth factor for strains with still earlier blocks. Salamon (28) isolated this precursor and identified it as 5-dehydroshikimic acid (DHS). The compound that in turn precedes DHS on the biosynthetic path was much slower to be recognized, since mutants blocked before it, in either *Escherichia coli* or *Aerobacter*, showed exceedingly little growth response. It was eventually found possible, however, to obtain from these mutants secondary derivatives that responded well to this compound and hence could be used for bioassay during its purification (12). Weiss (30) was then able to isolate the compound and identified it as 5-dehydroquinic acid (DHQ). Furthermore, mutants of *Aerobacter* that could respond to this substance were found to respond equally well to a well-known closely related compound, quinic acid. But *E. coli* mutants that responded to DHQ neither utilized nor accumulated quinic acid (12).

This is as far back as we have been able to penetrate by the method of searching for growth factors that could replace the aromatic supplement. At the other end of the chain, beyond shikimic acid, we have encountered three accumulated compounds that are completely devoid of nutritional activity for any mutant. One of these is found in filtrates of all mutants that accumulate shikimic acid, and on acid hydrolysis it was found to yield shikimic acid (11). It has been identified by Weiss as 5-phosphoshikimic acid. The second compound is accumulated by mutants that are blocked still later, and it too was found to yield shikimic acid on acid hydrolysis (11). This compound, provisionally termed Z1, is as yet unidentified.

The third inactive compound, prephenic acid (PPA), is accumulated by mutants that require phenylalanine or phenylpyruvic acid for growth (7, 18) and also by some that require tyrosine or the corresponding  $\alpha$ -keto acid. It has been isolated and identified by Weiss and Gilvarg (31). PPA is a nonaromatic compound, and it has particular biochemical interest because it is the substrate of the actual aromatization step in phenylalanine biosynthesis, losing both  $\text{CO}_2$  and water to yield phenylpyruvic acid.

I should now like to describe some studies on the enzymes linking certain of these compounds. These enzymes can easily be obtained in cell-free solution after disrupting wild-type *E. coli* cells, either by grinding with glass powder or by sonic oscillation.

The enzyme of this series that has been most extensively studied, from a physiological point of view, is 5-dehydroquinase, investigated by Mitsuhashi (22). It removes a molecule of water from DHQ to form DHS, and its activity is readily measured by virtue of the fact that DHS has a high absorption peak at 234  $\text{m}\mu$ , whereas DHQ does not absorb light significantly at this wavelength. Activity is strictly proportional to enzyme concentration. The reaction is reversible, with the equilibrium constant

(DHS/DHQ=15) heavily in favor of DHS. The activity of a wild-type extract is approximately that required to account for the rate of aromatic biosynthesis implied by the observed growth rate of the culture.

Two aspects of this study are particularly relevant to our interests here. The first is the evidence that the reaction involves a single enzymatic step. Since the reaction is a dehydration, resembling the reactions catalyzed by fumarase and aconitase, it would be expected, like the latter two reactions, to involve a single enzyme; the only plausible alternative would seem to be a series of reactions involving interaction with a cofactor (for example, conjugation or change in level of oxidation), followed by dehydration and then reversal of the interaction with the cofactor. But no cofactor requirement, organic or inorganic, could be demonstrated. The enzyme has been purified about tenfold by treatment with  $MnCl_2$ ,  $(NH_4)_2SO_4$ , and calcium phosphate gel. The product thus obtained, even after treatment with an anion-exchange resin to remove bound cofactors, was active without cofactor addition, and was equally active in tris-(hydroxymethyl)-amino-methane hydrochloride buffer or in potassium phosphate buffer. Hence, even though 5-dehydroquinase is still far from crystalline, its lack of a demonstrable cofactor requirement furnishes strong evidence that the reaction is mediated by a single enzyme.

The other aspect of this work that I should like to mention is that even though dehydroquinase activity is regularly demonstrable in extracts of the wild type, no activity could be demonstrated in extracts of mutants that were blocked in this reaction. The sensitivity of the assay was such that one would have detected the enzyme in a concentration 1/300 that of the wild type. Furthermore, mixtures of wild-type and mutant extracts showed precisely the same activity as the wild-type extract alone. This finding allows one to reject the possibility that the inactivity

of the mutant extract might be due to presence of an inhibitor or to absence of an essential activator; for, in order to account for the results of the mixture experiments, either an inhibiting or an activating factor would have to be stoichiometrically attached to the enzyme, and such a difference between two extracts would not be operationally distinguishable from the presence of active enzyme molecules in one and their absence from the other. We can therefore conclude that the genetic block between DHQ and DHS is indeed associated with inability of the mutant cell to make the enzyme for that reaction, at least in active form. Furthermore, the loss is specific; mutants blocked in various earlier or later reactions of the aromatic sequence have yielded normal or sometimes even increased amounts of dehydroquinase.

The enzyme that catalyzes the next reaction in this chain, the reduction of DHS to shikimic acid, has been similarly studied in extracts by Yaniv and Gilvarg (32). The enzyme is specific for triphosphopyridine nucleotide (TPN) as hydrogen carrier, and the reaction is reversible. There is strong evidence that this reaction also is catalyzed by a single enzyme, and this enzyme has no demonstrable cofactor or metal ion requirement other than TPN. As in the previous case, the wild type yields the enzyme in about the concentration expected from the growth rate; mutants blocked in this reaction yield no enzyme activity; and the addition of mutant extract has no effect on the activity of wild-type extract.

It might be noted at this point that both these enzymes have been found present in extracts of a variety of microorganisms and plants that can synthesize their own aromatic amino acids, and absent from an animal tissue which cannot synthesize these compounds. This path is therefore widely distributed in nature, and may well be the only path existing for the biosynthesis of this group of benzenoid compounds.



Let us return to quinic acid, which is a growth factor for certain *Aerobacter* mutants but not for any available *E. coli* mutants. The enzyme that catalyzes the oxidation of this compound to DHQ in *Aerobacter* has been extracted from the wild type by Mitsuhashi and has been called quinic dehydrogenase (23). It is specific for diphosphopyridine nucleotide (DPN), in contrast to the corresponding enzyme that interconverts DHS and shikimic acid, already noted to be TPN-specific. Quinic dehydrogenase is much more restricted in its distribution than are the two enzymes previously described; it could not be detected in extracts of wild-type *E. coli* or in various other microbial and plant extracts that did contain these other enzymes.

The enzymes concerned with phosphoshikimic acid and with compound Z1 have not yet been investigated. The enzyme that converts prephenic acid to phenylpyruvic acid has been shown to be present in wild-type extract, absent from an extract of a mutant blocked in this reaction, and present in the expected amount in a mixture of the two extracts (10, 31). It has not been purified, but on structural grounds it seems probable that only a single enzyme would be required to catalyze the reaction. Finally, Rudman and Meister (27) have described a transaminase from *E. coli* that converts phenylpyruvic acid and several other  $\alpha$ -keto acids to their corresponding  $\alpha$ -amino acids; no mutant deficient in this enzyme has yet been recognized.

And now, what criteria shall we use to decide on the metabolic role of each of these compounds? Historically, the first criterion to be used with mutants, and the only one used for a number of years, was growth-factor activity. Accordingly, one was hesitant to accept as an intermediate a compound that could not support growth, or even one that did so slowly or only at excessive concentrations. There has been increasing evidence, however, that growth-factor activity is not a necessary condition for function as an essential intermediate. (This point is discussed later.)

Furthermore, it is not a sufficient condition. For example, para-nitrobenzoic acid can effectively replace para-aminobenzoic acid as a growth factor for mutants of *Neurospora* (29) or *E. coli* (10), but only because the cells can non-specifically reduce nitro groups to amino groups. In such a case, even if one used isotopes to show that growth factor A was incorporated into cell constituent B, one would have demonstrated only that A *can* serve as a precursor of B, but not that A is an essential precursor of B, that is, an obligatory intermediate in the synthesis of B by the wild-type organism growing on a minimal medium.

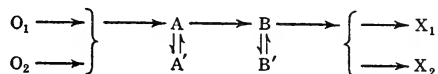
Accumulation of a compound by an auxotrophic mutant has seemed to offer more significant evidence that the compound is a true intermediate. But this property, too, is clearly not a necessary condition.<sup>2</sup> Furthermore, it is not a sufficient one. Some accumulated compounds are unstable and give rise, for example, to colored products that are very unlikely to be participants in biosynthesis (3, 21); and some accumulated intermediates appear to become conjugated, in a process akin to what in mammalian biochemistry is called detoxification (33).

We come, then, to enzymatic criteria. If a compound has nutritional activity, it must either be, or become spontaneously converted to, the substrate of some enzyme in the cell. Similarly, if a compound is accumulated, it must either be the product of some enzyme or be spontaneously formed from such a product. In consequence, the demonstration of such an enzyme does not alone, any more than

<sup>2</sup>For example, when an intermediate is common to several paths (as with glutamic acid), a block in its entrance to one of those paths would not be expected to cause its accumulation. Even with compounds restricted to a single path, the level of accumulation reached varies widely from one compound to another and also depends strikingly on the conditions of growth; it is sometimes not detectable. In general, accumulation of intermediates by mutants has been used by students of intermediary metabolism simply as a generous gift of the gods. The mechanisms responsible for such accumulation and, even more important, for its absence in the wild type constitute a major problem for future investigation.

the isotopic evidence for incorporation already mentioned, show that the compound is an essential intermediate. But if the enzyme that forms or utilizes the compound can be shown to be present in extracts of the wild type and absent from extracts of a mutant that is blocked in the biosynthetic chain in question—and if the enzyme assay is sensitive enough so that the observed difference between the two extracts is large—then one can begin to feel confident that he is dealing with an essential intermediate.

One more possibility, however, must be considered: that the true intermediates might be unstable or conjugated compounds, never present in more than trace amounts. The compounds that are detected as growth factors or as accumulation products would then be merely cast-off derivatives, pallid reflections of the living reality, just as, in Plato's parable of the cave, objects sensed in the world around us were considered to be mere shadows of an ideal world of true reality. Such a scheme might be represented as follows:



A and B would represent the true intermediates, and A' and B' the parallel side products. An auxotrophic mutant blocked between A and B would be unable to convert not only A to B, but also A' to B'. A distinction can be made, however, as Adelberg has pointed out (2), on the basis of the fact that a single enzyme could convert A to B, whereas three enzymes would be required to convert A' to B'.<sup>3</sup> We are led, therefore, to the following conclusion: that essential intermediates can be recognized at present with maximal certainty only in pairs, by showing (a) that the wild type (either cell or extract) can convert these

<sup>3</sup> We are referring here to substances (A and A') that are not in rapid spontaneous equilibrium with each other.

compounds (A and B) into one or more essential metabolic products ( $X_1, X_2 \dots$ ); (b) that a single enzyme that converts A to B is demonstrable in extracts of the wild-type organism;<sup>4</sup> and (c) that this enzyme is absent or inactive in the extracts of a mutant strain that has lost the ability to form  $X_1, X_2 \dots$  from the source materials  $O_1, O_2 \dots$ .

These criteria can be extended, of course, to include chemical rather than genetic elimination of the activity of an enzyme, provided that the effect of the inhibitor is restricted to a single enzyme.

Accepting these criteria, I think we can feel quite certain, on the basis of the enzymatic evidence presented, that DHQ, DHS, and shikimic acid are true normal intermediates in aromatic biosynthesis, and almost certain that PPA and phenypyruvic acid are normal intermediates in the synthesis of phenylalanine. The structure of PPA also suggests it as a likely precursor of tyrosine, and this possibility is supported by the fact that PPA is accumulated by some tyrosine auxotrophs. Until an enzymatic connection between PPA and tyrosine is established, however, we cannot be sure of this path.

The positions of phosphoshikimic acid and compound ZI are uncertain, since there are as yet no enzymatic studies on these compounds. Accumulation studies have already shown that ZI arises after shikimic acid and phosphoshikimic acid, but it may be either a direct member of the chain or a side product. Phosphoshikimic acid I would pro-

<sup>4</sup> Though in many cases an enzyme can carry out the same type of reaction on a variety of substrates, each of these usually gives rise to a different product; hence the assumption is made here that the essential enzyme that forms B from A can form B only from A. But this assumption may conceivably not be true (compare aconitase, a presumably single enzyme that can form aconitate from either citrate or isocitrate). It is therefore desirable to strengthen the evidence that A and B are the true, physiological substrate and product of an essential enzyme by showing further that they in turn are formed and utilized, respectively, by other essential enzymes in the same biosynthetic sequence.

visionally consider a side product, simply on the basis of the fact that among our 59 mutants blocked somewhere from before DHQ to after compound Z1, not one has been blocked between shikimic acid and phosphoshikimic acid.

With respect to quinic acid, I have even more serious reservations, despite its growth-factor activity for an *Aerobacter* mutant (12) and also for a *Neurospora* mutant (15). These reservations were originally based on two facts: (a) we could not obtain an *Aerobacter* auxotroph blocked between quinic acid and DHQ, and (b) the *E. coli* mutants that responded to DHQ neither responded to nor accumulated quinic acid; and if these strains had been blocked between the two compounds, they would have been expected to accumulate quinic acid. The case against this compound is now very much strengthened by the fact that quinic dehydrogenase, though present in *Aerobacter*, is absent from many other species, including *E. coli*, that do possess other enzymes of this path. Since *Aerobacter* has the same path as these other organisms beyond DHQ, it very likely also has the same path leading up to DHQ. Its enzyme for converting quinic acid to DHQ would then have to be regarded as an adventitious one, not essential for aromatic biosynthesis from glucose. This problem can be definitively settled when the enzymatic step before DHQ is worked out in *Aerobacter* and *E. coli*.

I should like to say a few words on the subject of growth-factor activity. Although this property has directed us to some of the compounds under investigation, we now blithely disregard it in assigning to each compound its place either at the table or in the corner. We accept prephenic acid, though it has no activity; we reject quinic acid, though it has activity. How can we account for the inactivity of a true intermediate?

One example is based on the fact that all mutants blocked immediately before shikimic acid show little response to this compound. The reason is that these strains accumulate

DHS, and this compound has been found to inhibit competitively the utilization of its own product, shikimic acid (6). But this is probably a special case; I suspect that inactivity of an externally added substrate is more frequently based on a permeability problem, or, more generally speaking, on inaccessibility of the compound to its enzyme within the cell. Since this explanation for inactivity, when offered by a biochemist, is generally shrouded with apologies for its *ad hoc* character, I should like to refer briefly to some recent findings in the aromatic series that offer more positive support for this view.

These findings are based on the behavior of an *Aerobacter* mutant blocked before DHQ. This strain shows a rather slow response to shikimic acid or DHS, and an even slower, barely detectable response to DHQ. But from this auxotroph one can select a secondary mutant that grows rapidly on DHQ; and this strain is then found to grow faster on DHQ than on DHS or shikimic acid, even in high concentrations (12). The enzyme studies described have convinced us, however, that (a) DHQ is an obligatory intermediate in the wild type growing on glucose, and (b) in functioning as such an intermediate (or as a growth factor in an auxotroph), it must be transformed into DHS. Furthermore, the enzyme that effects the transformation of DHQ to DHS is found in just as high a concentration in the primary auxotroph as in the wild type (24), even though the auxotroph can hardly grow on DHQ.

We are therefore forced to conclude that in the primary auxotroph, externally added DHQ cannot easily get to its enzyme, and in the secondary mutant this barrier has been eliminated. Furthermore, since this secondary mutant grows faster on DHQ than on its product, DHS, it seems clear that in this strain a partial accessibility barrier is still present for externally added DHS, in contrast to the DHS formed endogenously from added DHQ.

Although these findings do not constitute direct evidence

for an accessibility barrier, the indirect evidence they furnish is strong. Studies on this aspect of cell physiology are still in a primitive state; there is reason to hope that in investigation of this important area, mutants with altered accessibility will be of great help, just as auxotrophic mutants have been in analyzing biosynthetic paths. To point out how complex accessibility problems may be, I should like to note that the barrier to DHQ just described has been observed on the usual medium, in which glucose is the carbon source. If the glucose is replaced by xylose or succinate the barrier disappears, and the primary auxotroph and its secondary derivative then respond equally well to DHQ; but under these conditions, addition of a trace of glucose restores the barrier (12)!

To bring the aromatic story up to date, I should like to mention that a fusion of enzymatic and nutritional studies with mutants has made it possible to trace this path farther back than we were able to go by nutritional studies alone. In this work, my colleague, Edwin Kalan, has recently shown that mutants blocked before DHQ accumulate a precursor, "compound V." This substance, though without demonstrable growth-factor activity, could be recognized through its enzymatic conversion to DHS by extracts of mutants blocked after DHS. Furthermore, in collaboration with David Sprinson of Columbia University, he showed that these extracts also formed DHS from various phosphorylated carbohydrates. The whole early part of the path of aromatic biosynthesis is therefore now available for enzymatic analysis.

Finally, I should like to point out that the criteria proposed for recognizing an essential intermediate are not restricted to biosynthetic reactions. An extension to degradative reactions is useful, for though many of these reactions have been exhaustively studied, it has not been possible, in general, to assess definitively their physiological importance

by using enzymatic methods alone, or even in combination with isotopic methods. Let us consider the tricarboxylic acid cycle, whose energy-yielding, degradative aspects have been emphasized in the past, though its importance in the biosynthesis of glutamic acid and aspartic acid and their derivatives has only recently received more attention. Despite the fact that all the enzymes of this cycle have been demonstrated in *E. coli* and in various other microorganisms, there is no general agreement as to whether this cycle plays an essential or even an important role in the oxidation of acetate by these organisms.

To study this question, Gilvarg and I have used the methods described for biosynthetic reactions, employing a glutamic acid auxotroph of *E. coli* that turned out, on enzymatic analysis, to lack the condensing enzyme for forming citrate from acetyl-coenzyme A and oxaloacetate (14). It was found that this mutant not only required  $\alpha$ -ketoglutarate or glutamate as a growth factor (when growing on glucose as a carbon source), but also had completely lost the ability to use acetate as a carbon source or to form  $\text{CO}_2$  from acetate. Yet when *E. coli* derives its energy from acetate, the formation of  $\text{CO}_2$  is obligatory.<sup>5</sup> Hence, in terms of the criteria proposed, we have shown that absence of the citrate-condensing enzyme is necessarily associated with inability to form from acetate two obligatory metabolic products ( $\alpha$ -ketoglutarate and  $\text{CO}_2$ ) which the wild type can form. We can therefore conclude that the tricarboxylic acid cycle is essential in *E. coli* not only for biosynthetic purposes, but also for the complete oxidation of acetate. I believe this is the first conclusive demonstration of the latter point; it provides an excellent example of the sharpness of the tools furnished by microbial mutants.

<sup>5</sup> The possibility of succinate formation by back-to-back condensation of acetate was eliminated by the fact that this mutant, though unable to oxidize acetate, could still oxidize succinate.



To summarize their biochemical implications, the enzymatic studies described here have convinced us that some (but not all) of the compounds accumulated by aromatic auxotrophs are true essential biosynthetic intermediates. These results have therefore increased our confidence that most of the compounds accumulated by other mutants will have similar metabolic significance. Furthermore, a combination of enzymatic, nutritional, and accumulation studies, applied to mutants, has led us to previously inaccessible intermediates. Also, such an approach has made it possible to define more accurately the physiological role of the tricarboxylic acid cycle. In general, it appears that nutritional studies on mutants, like isotopic studies, have provided most valuable exploratory tools in the search for biosynthetic intermediates; that the identification of compounds accumulated by mutants, even when these compounds are devoid of nutritional activity, is a valid extension of this approach; and that enzymatic comparisons between mutants and the wild type can offer the most rigorous proof presently possible that a compound is an essential intermediate on a metabolic path.

To summarize their genetic implications, the enzymatic studies described here, and similar studies on other enzyme systems in this (19) and other laboratories (13, 25, 27), have shown that Beadle and Tatum were correct in their original assumption that an auxotrophic mutant lacks a specific enzyme. I believe we can feel confident that only an exceptional auxotrophic mutant requires a substance for some other reason: for example, the inhibition rather than the absence of an enzyme (34), or the destruction of the substance by an exaggerated side reaction (17).

There has not been time to describe other enzyme studies that have thrown a little further light on the relation between a gene and either the structure or the amount of the corresponding enzyme. With respect to enzyme structure,

the most direct evidence so far is furnished by the findings of Maas (20) and of Horowitz (16), who have shown that some mutants produce a qualitatively altered enzyme—one that performs the same reaction as the normal enzyme, but is much more sensitive to heat denaturation. With respect to the *amount* of the corresponding enzyme (or rather of its activity), we have shown with Mitsuhashi that in a group of aromatic auxotrophs the incompleteness of the block, as inferred from growth requirements (5), is directly related to the amount of enzyme (5-dehydroquinase) extractable from the cell (24).

I have not touched on the deeper problems raised by the one-gene-one-enzyme hypothesis: for example, how in some cases mutations at different loci block the same reaction; how mutations of modifier genes can restore a blocked reaction; whether all genes have the same kind of relation to protein synthesis as the genes that are altered in auxotrophic mutants; how directly or indirectly, and by what mechanisms, the structural specificity of a gene is translated into terms of structure and amount of an enzyme; how these mechanisms interact with inductive (“adaptive”) environmental effects on enzyme formation; whether the loss of activity of an enzyme in a mutant might be a special case of formation of an altered protein—one so drastically altered as to be no longer recognizable as an enzyme. In a sense, it is no longer profitable to argue about the validity of the one-gene-one-enzyme theory; we have all come to accept, consciously or unconsciously, the idea of a close connection between individual genes and individual enzymes. The problem now is that of analyzing the detailed mechanisms underlying this connection; and this genetic problem merges into other disciplines. These include physiology (for example, study of the environmental factors that induce or inhibit enzyme formation) and biochemistry (for example, study of protein synthesis and nucleic acid function).

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PART **2**

**METABOLISM OF  
MICROORGANISMS**



## Chapter 5

# *Progress and Problems in Bacterial Metabolism*

By H. A. BARKER

Bacterial metabolism is a very large subject. The chemical reactions in a single bacterium that are responsible for substrate accumulation, oxidative or fermentative metabolism, protein and enzyme synthesis, structural organization, and conversion of chemical energy to mechanical work cannot be explained by all our present biochemical knowledge. Furthermore, in a consideration of bacterial metabolism, the subject matter is increased many fold by the great variety of organisms. The metabolism of each species must differ in some way from that of every other, since every morphological and physiological character of an organism must have a chemical basis.

In view of the complexity of the subject, I have decided to restrict this discussion to a few observations on the historical development of the knowledge of bacterial metabolism, with some mention of certain areas of research in which notable progress has been made and other areas in

which even superficial examination shows that much remains to be done.

A glance at the history of microbiology shows that the development of knowledge of bacterial metabolism has passed through several more or less distinct but overlapping phases, characterized by differences in the objectives, the points of view, and particularly the experimental methods of the investigators.

The first phase can be described as that of exploration. Led by such pioneers as Pasteur, Winogradsky, and Beijerinck, studies in bacterial metabolism were concerned with the role of bacteria in a great variety of naturally occurring chemical processes like the souring of milk, the formation of nitrate in the soil, and the accumulation of hydrogen sulfide in Dutch canals. During this period many bacteria were discovered, and most of the basic and now familiar facts of bacterial physiology were established. The more obvious nutritional requirements of bacteria were determined; the existence of aerobic and anaerobic, autotrophic and heterotrophic, chemosynthetic and photosynthetic types of metabolism was demonstrated. Most of the presently known physiological groups of bacteria were recognized, and their main substrates, catabolic products, and environmental requirements were established at least qualitatively.

The exploratory phase of microbiology, of course, did not come to a sudden close, but has continued with decreasing activity to the present time. Within the last twenty-five years, numerous notable contributions have been made. As illustrations, I shall mention only the brilliant studies of van Niel and his associates on the photosynthetic bacteria, and of Hungate (11) and of Sijpesteijn (22), independently, on the cellulose-fermenting bacteria of the bovine rumen. These and other studies have shown that the exploratory phase of microbiology is by no means past, although it no longer occupies the center of interest.



About 1925, after the main physiological groups of bacteria had been discovered and their more conspicuous chemical activities had been determined, a new interest began to develop among microbiologists, that of finding out something about the chemical mechanisms of oxidative and fermentative processes. This interest was greatly stimulated by the work of the biochemists Harden, Neuberg, Meyerhof, and others, on alcoholic and lactic fermentations and respiratory mechanisms in yeast and muscle. Kluyver and his associates in Holland and Fred and Peterson and others in the United States gave particular attention to the comparable bacterial processes, including the lactic, propionic, butyric, acetone-butanol, and other fermentations.

The main method used by the microbiologists was that of the balance experiment. Careful quantitative analyses were made of the products of fermentation caused by growing bacterial cultures. On the basis of the data obtained with various substrates such as glucose and pyruvate, an attempt was made to determine the most probable of several alternative mechanisms which could be suggested to account for the fermentation products. This method yielded a great deal of quantitative information of permanent value about bacterial fermentations. It also resulted in the elimination of several theories of a speculative character concerning the mechanism of fermentations that were inconsistent with the quantitative data. The balance experiment method, however, was incapable of solving the problems of intermediary metabolism because it provided too little information concerning what actually goes on within bacterial cells, and left too much to speculation based mainly on a limited knowledge of the enzymatic reactions occurring in yeast and muscle.

One of the very important developments on the conceptual level at this time was the recognition, particularly by Kluyver and his associates, of a general similarity in the metabolic processes of various microorganisms. During the

exploratory period a great variety of metabolic processes were discovered which on superficial examination appeared to be wholly unrelated. For example, most microbiologists of thirty years ago saw little connection between the utilization of oxygen by the acetic acid bacteria for the conversion of alcohol to acetic acid and the formation of hydrogen sulfide by the sulfate-reducing bacteria. Kluver pointed out that both oxygen and sulfate are reducible compounds and that they serve essentially the same function, as oxidizing agents, in the metabolism of these two types of bacteria. Kluver also perceived that the chemical reactions catalyzed by bacteria are generally oxidation-reduction processes in which some compounds are oxidized and others are reduced in a series of simple and chemically intelligible steps. His analysis of the available data on hexose decomposition by different bacteria led to the view that in most cases the process consists of a conversion of the sugar to two  $C_3$  compounds, which in turn are converted to  $C_2$  and  $C_1$  compounds. These various pieces of the hexose molecule are then transformed by oxidation, reduction, and condensation reactions to the observed products of catabolism. Our knowledge of bacterial carbohydrate metabolism has greatly expanded in recent years, and numerous changes have been required in this simple picture. Nevertheless, the concept of an underlying unity in biochemistry, first developed in some detail by an analysis of the extensive information on bacterial metabolism, has become a cornerstone of our science.

The severe limitations of the chemical balance experiment method for the study of intermediary metabolism in bacteria were soon recognized, and a search for better methods was stimulated. During the last twenty years several new or improved methods have been developed which are largely responsible for the current rapid expansion of knowledge in this field.

One of these is the tracer method. Use of the stable and

radioactive isotopes of carbon, nitrogen, phosphorus, and other elements has enormously simplified certain kinds of metabolic studies. Tracer methods frequently permit the direct observation of chemical processes that previously could be detected and studied only, if at all, by much more indirect and laborious procedures. For example, the carbon isotopes made it possible to determine the fate of individual carbon atoms during the metabolic transformation of a multicarbon compound. This supplied new information about the chemical reactions in living bacteria and enzyme systems that could be used to determine the validity of proposed metabolic pathways. Tracer methods also permitted the study of the utilization of compounds that normally accumulate during metabolism. In this way the surprising discovery was made that several common end products of bacterial metabolism like acetate and carbon dioxide, previously regarded as stable and inert, are in fact intermediates that can be further converted by many bacteria into a variety of catabolic products and cellular constituents.

The balance and tracer methods give a picture of the over-all chemical transformation in bacterial metabolism and also provide a guide to the chemical mechanisms involved. These techniques are limited, however, by the fact that in living bacteria the metabolic processes are so perfectly coordinated that it is difficult to separate and identify the individual reactions. Also, intact bacteria are impermeable to many compounds of biochemical importance such as phosphate esters and coenzymes. Therefore a detailed insight into the cellular chemical mechanisms, which are responsible for substrate decomposition and the synthesis of essential cellular components, requires the use of cellular extracts and enzymatic techniques. This has been obvious for a long time, ever since the discovery of the glycolytic mechanism. But, the required combination of microbiological and enzymological know-how was scarce, and so,

until recently, progress in the analysis of enzymatic mechanisms in bacteria has been relatively slow. An additional impediment for the microbiologist was the lack of good methods for preparing enzymatically active cell-free extracts from bacteria. This difficulty has now been largely overcome by development of the alumina grinding (16), sonoration, and other techniques of preparing bacterial extracts. As a result of this, together with the continually increasing interest in intermediary metabolism and the development of basic knowledge of enzymatic mechanisms, research on bacterial enzyme systems has expanded tremendously during recent years. The new generation of microbiologists and biochemists is now vigorously attacking the many meaty problems of bacterial metabolism.

Nutritional methods have also been applied extensively for the study of the synthetic capacities and biosynthetic mechanisms of bacteria. The study of bacterial nutrition has led to the detection and isolation of a number of compounds or growth factors which are essential components of the enzymatic machinery of all or most living organisms, but which are specifically required as nutrients only by those organisms that are unable to synthesize them from simpler substances. The nutritional approach to the study of the biosynthetic pathways of amino acids, growth factors, and other cellular constituents has been enormously stimulated by the discovery of methods of effectively blocking the chemical reactions of the cell so as to cause the accumulation of various compounds in the chain of synthetic reactions. Such blocking can be achieved by the isolation of appropriate biochemical mutants by modification of the methods first developed by Beadle and Tatum, or by the use of more or less specific chemical inhibitors. Much of our present knowledge of biosynthetic pathways in bacteria is based upon the use of these and related methods.

Many other new methods and techniques have smoothed the way for the student of bacterial metabolism. Of these,

I shall draw your attention only to the so-called simultaneous adaptation technique, developed independently by Stanier (24) and by Karlsson and Barker (12) in the United States, and by Suda and others (25) in Japan, which can often be used to map out the approximate sequence of steps in a biochemical process. Stanier has applied this method with great success in the study of the bacterial oxidation of aromatic compounds.

Finally, I must mention the techniques of column and paper chromatography, which have largely replaced earlier methods for the detection, isolation, and estimation of small quantities of all kinds of biologically important compounds. No laboratory for the study of bacterial metabolism is complete these days without facilities for chromatographic analysis.

The rapid expansion of knowledge of bacterial metabolism has been attributable only in part to the work of microbiologists. Many biochemists, who have little interest in microbiology *per se*, have come to realize that microorganisms provide extremely convenient experimental material for the study of basic biochemical phenomena. The common objective of microbiologists and biochemists is to obtain a detailed understanding of the chemical mechanisms that are responsible for the formation of living cells from simple organic and inorganic nutrients. Since bacteria are extremely complicated chemical systems that carry out within the space of a single cell thousands of separate yet integrated reactions, obviously a multitude of problems await the student of bacterial metabolism. I shall mention only a few of these problems.

### **Bacterial Nutrition**

First, it is necessary to know the minimal nutritional requirements of the organisms. Here our knowledge is fairly complete for those few bacterial species whose nutritional

requirements have been examined carefully. A great many bacteria, however, have not been studied adequately from the nutritional point of view. The general requirements of these organisms undoubtedly will be similar to those already known. But from past experience, it can be anticipated that at least a few of these bacteria will ultimately be found to require new types of growth factors. Within recent years several such substances have been discovered through the study of microbial nutrition. One is lipoic acid, which has been shown to play an essential role in the oxidation of  $\alpha$ -keto acids. Another is ferrichrome, an iron-containing polypeptide derivative isolated by Neilands (18) from a smut fungus, *Ustilago sphaerogena*, and found to stimulate the growth of a soil bacterium. A third is a disaccharide of galactose and N-acetyl-glucosamine, recently shown by Zilliken and others (30) to have growth-promoting activity for *Lactobacillus bifidus*. Undoubtedly many more new compounds of biological importance will be found by further systematic investigation of bacterial nutrition.

### *Respiration and Fermentation*

Next are the problems of the oxidation and fermentation of the major substrates. Much is known about the compounds utilized and the major excretory products. A good beginning has been made in determining the chemical steps by which these energy-yielding reactions proceed. Yet here also much remains to be done. Only recently, for example, have we begun to discover some of the pathways by which bacteria convert glucose to three carbon compounds (6, 29).

#### GLUCOSE DECOMPOSITION

Until a few years ago, it was commonly accepted that sugar decomposition in bacteria occurs by the glycolytic mechanism. As late as 1952, Elsdon (3), in a review of exist-

ing knowledge on bacterial fermentations, was able to start with the thesis that "the primary mechanism found in muscle and yeast, the operation of which results in the formation of pyruvic acid, is found also in those bacteria which ferment carbohydrates." This thesis is no longer tenable, and no one should assume the existence of a glycolytic mechanism in a bacterium without clear and specific proof.

*Gluconic Acid Pathway.* The first example of a non-glycolytic pathway of glucose oxidation by bacteria was discovered long before glycolysis was known.

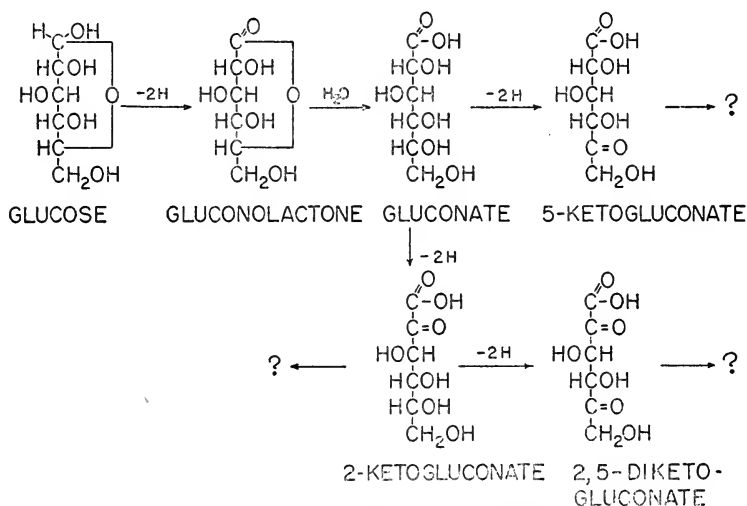


FIGURE 1. Gluconic Acid Pathways

In 1886, Brown found that species of *Acetobacter* oxidize massive amounts of glucose to gluconic acid. Not long afterward, Bertrand obtained evidence for the further oxidation of gluconate to 5-ketogluconate by *Acetobacter xylinum*. Recently Katznelson, Tannenbaum, and Tatum (13) studied glucose and gluconate oxidation by another species, *Acetobacter melanogenum*, which is characterized by formation of a brown water-soluble pigment. The investigators have provided convincing evidence that this organism

oxidizes gluconate to 2-ketogluconate, which is then further oxidized to 2,5-diketogluconate (Figure 1). The last compound is chemically unstable and is converted, in part, into the characteristic brown pigment of this species. 2-ketogluconate also is a major product of glucose oxidation by several *Pseudomonas* species.

The unusual feature of this type of glucose oxidation is that it involves only nonphosphorylated compounds. Almost no information is available at present about the further steps in the oxidation of the various ketogluconates or about the role of these compounds in the energy metabolism of the bacteria. Enzymes are known that phosphorylate gluconate and 2-ketogluconate, but it is not known whether such phosphorylation is an obligatory requirement for the complete oxidation of these compounds.

*Ribulose Phosphate Pathway.* The existence of another nonglycolytic mechanism for the direct oxidation of carbohydrate via 6-phosphogluconate by animals and yeasts was established first by the work of Warburg, Lipmann, and Dickens in 1935-36. The specific reactions involved in this process (Figure 2) have recently been worked out by Horecker, Racker, Cohen, and others (10).

Glucose is first phosphorylated and the resulting glucose-6-phosphate is oxidized to 6-P-gluconate. This in turn undergoes an oxidative decarboxylation to carbon dioxide and ribulose-5-phosphate. Although it has not been identified, 3-keto, 6-phosphogluconate has been postulated as an intermediate in the latter reaction. The ribulose-5-phosphate isomerizes to give ribose-5-phosphate. The upper two carbons of ribulose-5-phosphate are next transferred to carbon atom 1 of ribose-5-phosphate to give one molecule each of glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate (Figure 3). These compounds then react in such a way that the first three carbon atoms of the sedoheptulose-phosphate are transferred to triose phosphate, giving fructose-6-phosphate and a tetrose phosphate. There is now some evidence



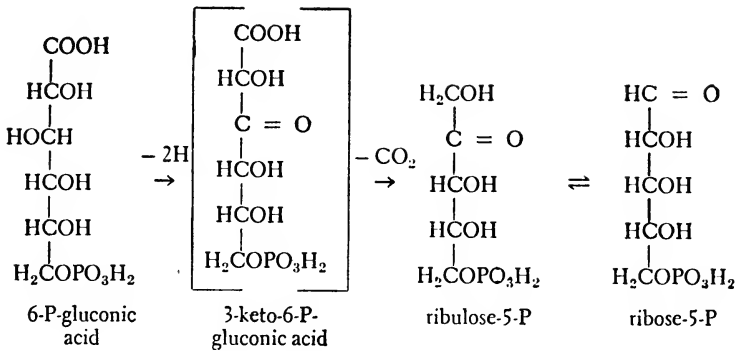


FIGURE 2. Ribulose Phosphate Pathway

that the latter can react enzymatically with another molecule of pentose phosphate to reform an additional molecule of hexose-phosphate and a triose phosphate. The net result of this cyclical series of reactions is the conversion of one molecule of hexose monophosphate to three molecules of carbon dioxide and one of triose phosphate.

It is worth pointing out that this type of sugar break-

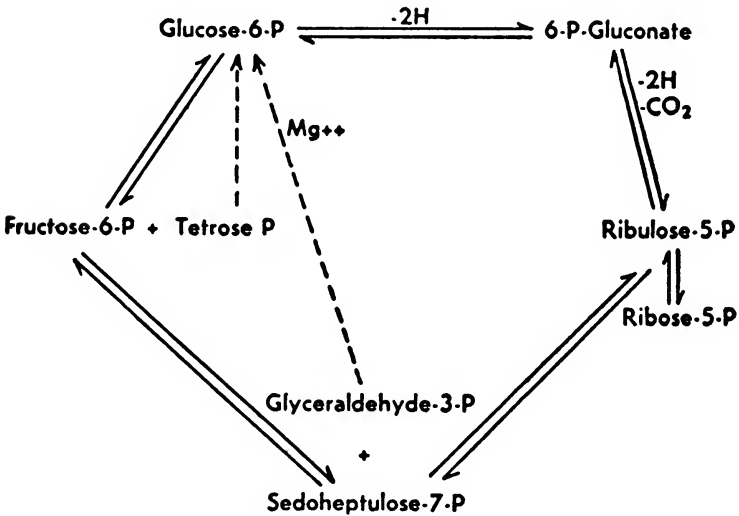


FIGURE 3. Formation of Glyceraldehyde-3-Phosphate and Sedoheptulose 7-Phosphate

down has special advantages to the cell because it makes available a variety of carbon chains of differing lengths. In fact, compounds containing one to seven carbon atoms are formed from the hexose molecule by a most ingenious system of group transfer reactions. The advantage of such an assortment of compounds to a cell engaged in the synthesis of a great number of more or less complex compounds is obvious. For example, the pentose phosphate can be used immediately for making ribonucleic acids and a variety of ribose-containing coenzymes. Probably each of the other compounds also serves as the starting point for some synthetic process.

Although much of the experimental work that has established this oxidative mechanism was done with yeast, liver, and plant enzymes, there is abundant evidence that some bacteria including *Escherichia coli* (21) and *Azotobacter vinelandii* (17) also utilize this mechanism of glucose oxidation.

A perhaps similar nonglycolytic mechanism, which has long been suspected to exist but which has only recently been demonstrated in a convincing manner, is found in the fermentative bacterium *Leuconostoc mesenteroides*. Gayon and Dubovag showed in 1894 that the fermentation of one mole of glucose by *Leuconostoc* species yields one mole each of lactate, ethanol, and carbon dioxide. The remarkable feature of this fermentation, from the point of view of the mechanism of sugar decomposition, is the constancy in the yields of the three products. Other bacterial glucose fermentations result in formation of the same compounds, but the ratio of lactate to ethanol, for example, is highly variable with pH and other environmental factors. With *Leuconostoc*, half of the sugar is always converted to lactate and half to ethanol and carbon dioxide. This result is inconsistent with the glycolytic mechanism, which requires both halves of the glucose molecule to follow a common path between glyceraldehydephosphate and pyruvate.

The existence of an unusual mechanism of glucose fermentation in *Leuconostoc* was clearly established by the tracer experiments of Gunsalus and Gibbs (8). The fermentation of glucose-1-C<sup>14</sup> and glucose-3, 4-C<sup>14</sup> showed (Figure 4) that the carbon dioxide originates from carbon atom 1 of glucose, the methyl group of ethanol from carbon 2, and the carbinol group from carbon 3. The lactate car-

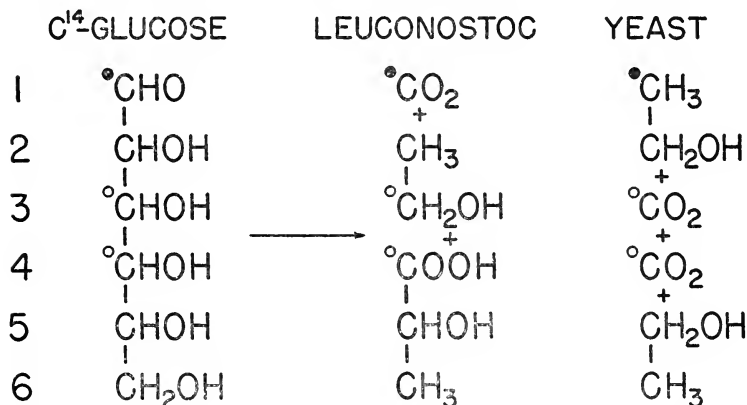


FIGURE 4. Glucose Fermentation by *Leuconostoc*, after Gunsalus and Gibbs (7)

bons, starting with the carboxyl group, are derived from glucose carbon atoms 4, 5, and 6. These results showed the expected lack of mixing of the carbon from the two ends of the glucose molecule, and also established an unusual origin for the carbon dioxide and ethanol. In alcoholic fermentation by yeast, carbon dioxide is derived from carbons 3 and 4 of glucose, and ethanol comes from carbons 1, 2, 5, and 6.

The experiments of DeMoss (2) with cell-free extracts of *Leuconostoc* provide additional evidence of a new mechanism. These investigators found that the enzymes aldolase and triose phosphate isomerase, which are essential to the glycolytic mechanism, are not present in these bacteria. The details of the mechanism of glucose breakdown by *Leuconostoc* have not yet been worked out, but there is con-

siderable evidence that glucose-6-phosphate and 6-phosphogluconate are involved, and the mechanism may be similar to the hexose monophosphate shunt mechanism in yeast.

*The 2-Keto, 3-Desoxy, 6-Phosphogluconate Pathway.* One of the best understood nonglycolytic mechanisms of glucose decomposition is that discovered in *Pseudomonas saccharophila* by Doudoroff and his associates. This organism is an obligate aerobe that normally oxidizes glucose to carbon dioxide without appreciable accumulation of intermediate products. Entner and Doudoroff (4) showed, however, that in the presence of suitable inhibitors (DNP and arsenite), cell suspensions oxidize either glucose or gluconate with the accumulation of two moles of pyruvate. A tracer experiment with glucose-1-C<sup>14</sup> showed that the carboxyl group of pyruvate is derived in part from carbon

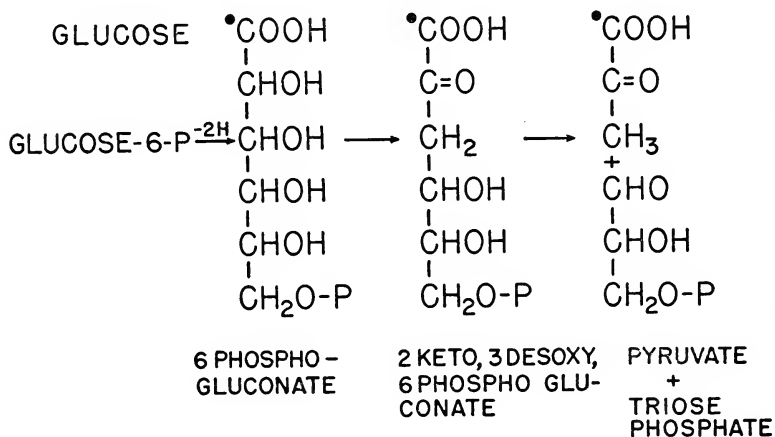


FIGURE 5. *Pseudomonas saccharophila* Pathway of Glucose Oxidation, after MacGee and Doudoroff (15)

atom 1 of glucose. Further enzymatic studies established that glucose is first phosphorylated and then oxidized to phosphogluconate. MacGee and Doudoroff (15) have shown that the latter compound undergoes an enzymatic rearrangement to yield a new compound, which has been iso-

lated as the crystalline sodium salt and identified as 2-keto, 3-desoxy, 6-phosphogluconate (Figure 5). This substance is then split enzymatically to pyruvate, which is derived from carbon atoms 1 to 3 of glucose, and 3-phosphoglyceraldehyde, derived from carbon atoms 4 to 6. The same reactions occur in *Pseudomonas fluorescens* (14).

A closely related sugar decomposition mechanism has been detected in a fermentative bacterium, *Pseudomonas lindneri*, by the tracer method. This organism carries out a typical alcoholic fermentation in so far as the products are concerned. The experiments of Gibbs and DeMoss (5)

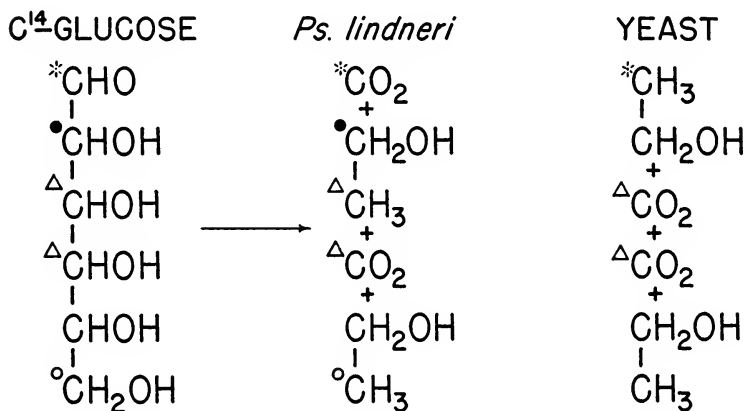


FIGURE 6. Alcoholic Fermentation by *Pseudomonas lindneri*, after Gibbs and DeMoss (6)

show, however, that the carbon dioxide is derived from carbon atoms 1 and 4 of glucose; the carbinol group of ethanol comes from carbons 2 and 5, and the methyl group from carbons 3 and 6 of glucose (Figure 6). This carbon distribution pattern is completely different from that obtained with yeast and is sufficiently similar to that observed with *Pseudomonas saccharophila* to suggest that both organisms use the same mechanism for converting glucose to three carbon units. But further work with cell-free extracts will be necessary to establish this possibility.

*Glycolytic Pathway.* After drawing your attention to these new types of sugar decomposition, I must not neglect to say that a few species of bacteria apparently still persist in using the old-fashioned glycolytic mechanism. I say "apparently" because with several of these bacteria the supporting evidence is not conclusive. The most extensive evidence for a glycolytic mechanism has been obtained with *E. coli* (3) and with *Streptococcus faecalis* (19). In particular, the formation and decomposition of fructose-1-6-diphosphate, the characteristic compound of the glycolytic pathway, has been demonstrated with these organisms. The occurrence of glycolysis in *Clostridium perfringens* is strongly indicated by the presence of aldolase, the enzyme converting fructose diphosphate to triose phosphate, which was demonstrated by Bard and Gunsalus (1). With *Clostridium thermoaceticum* and *Lactobacillus casei*, the distribution patterns of  $C^{14}$  in products obtained in the fermentation of  $C^{14}$ -labeled glucose are indicative of a predominantly glycolytic pathway, but detailed enzymatic studies are lacking.

The available information on glucose decomposition by bacteria may be summarized by saying that at least four distinctly different mechanisms are known, and some of these have recognizable variations. These mechanisms are not mutually exclusive, since at least two of the systems have been found in *P. fluorescens* and *E. coli*. Probably combinations of these systems will be found in many if not all bacteria. It is impossible at present to say whether these are the only pathways of sugar breakdown that exist in bacteria or to estimate which pathways are used more commonly. Clearly this is an area of research where much has been accomplished but where much still remains to be done.

#### OXIDATION OF PYRUVATE AND ACETATE

A further step in the decomposition of carbohydrate is the oxidation of pyruvate to acetate. This process has been

studied intensively with bacterial enzymes in several laboratories (7) and has been found to be unexpectedly complicated. The reaction involves the participation of no less than five coenzymes; namely, thiamine pyrophosphate, lipoic acid, coenzyme A, diphosphopyridine nucleotide (DPN), and adenosine diphosphate (ADP), and possibly as many apoenzymes. The role of each of the coenzymes has been fairly well established, although some details remain to be worked out. For example, it is not yet certain whether thiamine pyrophosphate and lipoic acid act entirely independently, or whether a compound of the two, lipoyl-thiamine pyrophosphate, is an essential component of the system. But it is probable that this and other controversial features of the mechanism of pyruvate oxidation can be resolved in the near future.

The mechanism of acetate oxidation in aerobic bacteria has also been a subject of great interest recently. Until 1948 it was generally assumed on the basis of very limited data that the well-known tricarboxylic acid (TCA) cycle is the main pathway of pyruvate and acetate oxidation. Then several observations strongly suggested that the cycle is not responsible for acetate oxidation (12). For example, cell suspensions of *Azotobacter agilis* were shown to oxidize acetate, whereas components of the TCA cycle, like citrate and  $\alpha$ -ketoglutarate, were not oxidized under the same conditions. In addition, numerous tracer experiments demonstrated that the oxidation of C<sup>14</sup>-labeled acetate by living bacteria does not always result in the labeling of TCA-cycle compounds that are added to the medium. Such experiments stimulated an extensive reinvestigation of the role of the TCA cycle by all available methods. As a result it has been definitely established that *Azotobacter*, *E. coli*, and several other bacteria possess the enzymatic machinery necessary to operate the TCA cycle. As far as I am aware, there is at present no clear-cut evidence for the existence of any alternative mechanism of acetate oxidation, such as the

frequently postulated dicarboxylic acid cycle in which acetate is converted directly to succinate. Furthermore, most of the earlier results that appeared to disprove the operation of a TCA cycle can now be satisfactorily explained on the basis of permeability barriers in the cell. It seems justifiable to conclude that the TCA cycle is a functional oxidative mechanism in some bacteria, although the existence of additional pathways of terminal respiration certainly cannot be excluded. This is essentially the same conclusion that could have been reached ten years ago, but it is now based upon a much larger body of factual information.

In addition to serving as an oxidative mechanism, the TCA cycle is useful as a device for providing a variety of reactive compounds containing four, five, and six carbon atoms that serve as starting points for the synthesis of essential cellular constituents, such as amino acids. For example,  $\alpha$ -ketoglutarate is the immediate precursor of glutamic acid, which can in turn be converted by *E. coli* via glutamic semialdehyde to proline or via N-acetyl glutamic acid and N-acetyl ornithine to arginine, as has been shown by Vogel (27).

#### FERMENTATION PRODUCTS

The anaerobic transformations of pyruvate and acetate have been studied even more extensively than the aerobic reactions. The pathways of formation of most of the common bacterial fermentation products, such as ethanol, acetate, propionate, butyrate, lactate, butanol, and acetone are fairly well understood, although a good deal remains to be done on the enzymatic level. It is also likely that different mechanisms are operative in the formation of some of these compounds in different bacterial species. This is known to be true for acetate, which is formed by several anaerobic bacteria from carbon dioxide by an entirely unknown mechanism.



As an example of the changing concepts of the chemistry of bacterial fermentations, I can tell you something about the formation of butyric acid. Early work on the origin of butyrate indicated that it is formed from a two-carbon compound usually derived from pyruvate. The identity of the two-carbon compound was long a matter of speculation. Acetaldehyde was suggested as a possibility. Several persons proposed that acetaldehyde might undergo an aldol condensation to give acetaldol, which might rearrange to yield butyric acid. But no convincing evidence could be obtained for this theory. Then in 1943, Koepsell and Johnson found that extracts of *Clostridium butylicum* convert pyruvate to acetyl phosphate rather than to acetaldehyde. This suggested that acetyl phosphate, a very reactive compound first discovered by Lipmann, might be the two-carbon precursor of butyrate. Support for this idea was obtained by Stadtman (23), who showed that cell-free extracts of *Clostridium kluyveri* convert acetyl phosphate and acetate more or less quantitatively to butyrate in the presence of a suitable reducing agent. Later work based on the discovery of coenzyme A by Lipmann, and of acetyl CoA by Lynen, has shown that acetyl CoA rather than acetyl phosphate is the immediate precursor of the four carbon derivatives that are ultimately converted to butyrate.

The reactions now known to be involved in the conversion of ethanol and acetate to butyrate by *C. kluyveri* are shown in Figure 7. The ethanol is oxidized to acetaldehyde and then to acetyl CoA. The carbohydrate-fermenting clostridia do not use ethanol or acetaldehyde, but form acetyl CoA directly from pyruvate. Two molecules of acetyl CoA are then condensed to acetoacetyl CoA which is converted by two reduction steps and a dehydration step to butyryl CoA. The latter reacts with acetate to give butyrate and at the same time regenerates a molecule of acetyl CoA, which again enters the cycle.

A noteworthy feature of this mechanism is that most of the reactions occur only while the carboxyl groups of the acids are in combination with the coenzyme. The rea-

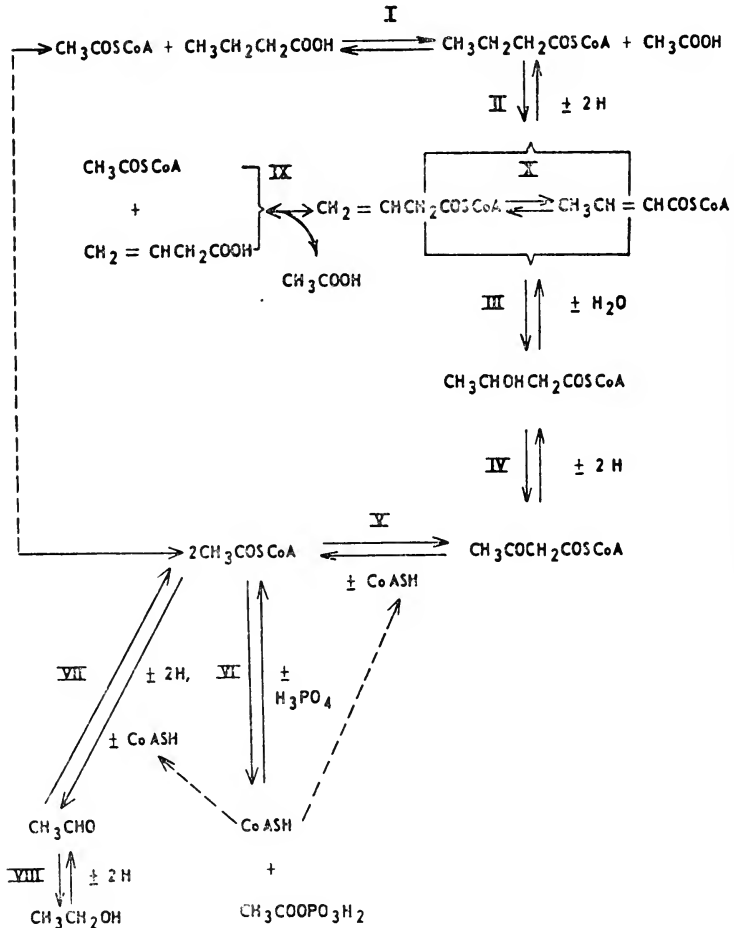


FIGURE 7. Scheme of Fatty Acid Oxidation by *Clostridium kluyveri*

son for this is not known, although it is probable that the large coenzyme molecule serves as a tool for attaching the substrate to the right part of the enzyme surface. Similar

coenzyme A substrate compounds have been found to be essential in other reactions catalyzed by bacteria. For example, Whitely (28) has shown that succinyl CoA and propionyl CoA are involved in the formation of propionate by the propionic acid bacteria, and Hayaishi (9) has found that malonyl CoA is an intermediate in the conversion of malonate to acetate by *Pseudomonas*.

Though our knowledge of the enzymatic reactions involved in butyrate synthesis is fairly detailed, it must be admitted that our understanding of the role of this process in the metabolism of *C. kluyveri* is still very deficient. We do not know, for example, what benefit the organism derives from the conversion of alcohol and acetate to butyrate, since the useful energy derived from the oxidation of the alcohol appears to be consumed again in the synthesis of butyrate, without leaving any energy available for other synthetic reactions.

### **Formation of Energy-Rich Compounds**

Another essential aspect of bacterial metabolism is the nature of the chemical mechanisms by which energy is generated and made available for synthetic purposes in the cell. We owe particularly to Lipmann the idea that compounds containing the so-called energy-rich phosphate bond represent a common currency for energy exchange in living organisms. More recently, as a result of the work of Lipmann, Lynen, and others, various thioesters, like acetyl CoA, also have been shown to play a prominent role in energy transfer.

The formation of such energy-rich compounds has been studied extensively in animals and yeast, and it has been found that they are usually formed in two ways, either by the oxidation of substrate molecules such as aldehydes and  $\alpha$ -keto acids, or as a result of the transfer of electrons by way of flavoproteins and the cytochrome system to oxygen.

An example of energy-rich phosphate bond formation in bacteria with a substrate molecule is the oxidation of acetaldehyde or pyruvate to acetyl phosphate. Only a relatively few reactions of this type, however, are known. Most of the energy-rich phosphate required to drive biosynthetic reactions in the oxidative metabolism of animals is generated by the electron-transporting mechanisms. In certain bacteria such mechanisms must have even greater significance in the economy of the cell. The autotrophic bacteria, in particular, obtain all their energy by the oxidation of such compounds as hydrogen and sulfur; obviously they cannot generate energy-rich compounds by substrate-linked reactions. They must be entirely dependent upon their electron-transporting systems for this vital function. As yet, however, virtually nothing is known about energy-rich phosphate-bond formation by such systems in either autotrophic or heterotrophic bacteria. Here then is an aspect of bacterial metabolism which obviously needs to be explored, and which could yield information of fundamental significance both to microbiology and to biochemistry.

Before leaving the topic of electron transport, I shall refer briefly to recent developments in the knowledge of bacterial cytochrome systems. The work of Smith and Chance, of Vernon and Kamen, and others has shown clearly that bacteria contain several types of cytochrome and cytochrome oxidase that differ markedly from the classical cytochrome components of heart muscle and yeast. Another new and unexpected development has been the discovery of relatively large amounts of cytochrome components in some obligately anaerobic bacteria like the sulfate-reducing bacteria (20) and some of the photosynthetic bacteria. The role of cytochrome in the sulfate-reducing bacteria is not yet known, though it presumably acts as an electron carrier between the oxidizable substrate and sulfate. Vernon and Kamen (26) have obtained evi-

dence that in *Rhodospirillum rubrum* the cytochrome system participates in the photosynthetic process, since the bacterial cytochrome is reduced by organic electron donors and is reoxidized by a light-generated oxidizing agent through the action of a specific cytochrome photooxidase. With these recent developments, a rapid extension of knowledge of the nature and function of bacterial cytochrome systems can be expected in the near future.

### ***Biosynthetic Pathways***

One of the most important aspects of bacterial metabolism, to which I have scarcely referred, is the synthesis of cellular constituents, such as amino acids, purines, proteins, and polysaccharides. Since many bacteria grow in simple media containing only one or a few organic compounds, it is obvious that they possess highly developed synthetic mechanisms. Time does not permit me to give even a superficial account of progress and problems in the analysis of biosynthetic pathways. Suffice it to say that elucidation of the mechanisms of biosynthesis is a major objective of current research in bacterial metabolism, and it undoubtedly will receive increasing emphasis as the simpler and more conspicuous problems of bacterial catabolism are satisfactorily solved.

I hope this brief survey of a few aspects of bacterial metabolism is convincing evidence that the subject is in a state of active development. As I have tried to emphasize, we are now in the midst of an attempt, started many years ago, to discover the chemical mechanisms whereby relatively simple nutrients are converted into living organisms. I say "organisms" rather than "bacteria" because even for microbiologists the real objective is to understand the chemical basis of life rather than the peculiarities of one or another bacterial species. A great deal has been learned about the dynamic aspects of biochemistry by the study of

bacterial metabolism, but a long and fascinating road must still be traversed before we can arrive at an adequate understanding of the chemical machinery of even the simplest of living organisms.

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## Chapter 6

# *Molds as Metabolic Models*

By JACKSON W. FOSTER

To persons cognizant of the enormous numbers and of the infinite heterogeneity of fungi, the futility of an attempt to discourse on the broad subject of metabolism of fungi is obvious. The vast majority of significant studies of fungal metabolism, however, have been done on "lower," or filamentous fungi (the molds). Consequently, the scope of this paper is automatically restricted; even so, only isolated aspects of the subject can be taken up effectively. My purpose is to reflect on the implications that certain studies on fungi have for issues which possess biological importance far beyond the immediate fact of their elucidation in fungi.

Before taking up the more technical features of this discussion, I should like to venture a few personal viewpoints relative to mold metabolism as a field of study. Frequently one sees evidence of an implicit assumption that training in technical mycology is a prerequisite for investigations on metabolism of fungi and that, lacking this, one had best avoid contending with these organisms. Though this assumption undoubtedly is valid for classical mycology,



where morphological, phylogenetic, and pathological researches demand a unique technology, it is equally true that investigation of the *activities* of fungi employs the equipment, the working methodology, the basic principles, and especially the experimental rationale which are characteristic of studies on *all* microorganisms, that is, the science of microbiology.

College-level bacteriology usually is the student's entrance to microbiology; and as nearly as I can determine, a conditioning to regard metabolism of fungi as a thing apart originates there. Although most early courses in bacteriology indoctrinate the student in the characteristic physiological features of organisms as diverse as yeasts, bacteria, and viruses, it seems that fungi are studied only so far as to enable the student to recognize them as obnoxious contaminants in his bacterial cultures.

It appears incongruous to delineate metabolism of bacteria from that of fungi, since the two overlap in so many respects and since each group displays, more than any other groups of microorganisms, a kaleidoscopic array of metabolic diversity. The concentration exclusively on metabolic activities of bacteria produces students whose microbiological vision is one-dimensional in a stereoscopic profession. In view of the homology between bacteria and fungi with respect to methodology, to roles in natural processes, to applied microbiology, and to exhibition of biological phenomena, it is paradoxical that special efforts are made to ignore, or at least to segregate, academic study of the fungi in the curriculum of the bacteriology major. For example, the three most recent and influential textbooks published in this country for use in courses in bacterial physiology carry no mention of the metabolic activities of fungi. Now, there may be good reasons for ignoring in a text on bacterial physiology the molds, the yeasts, the algae, the actinomycetes, and others. But should we attempt to justify this to the student simply on the

basis that the principles of bacterial physiology can be applied to all other organisms?

The unilateral approach in bacterial physiology courses would be at least understandable if exposure to an independent course in mold metabolism were available for or required of the bacteriology major. To the best of my knowledge, only a handful of colleges in the United States present course coverage of this subject matter to a degree comparable to the coverage of bacterial metabolism.

From the flavor of the foregoing, it should not be construed that I would advocate pursuit of the fungi with the object of characterizing their activities in chemical symbolism. To the contrary, we need more and more to utilize the chemical reactions, to interpret the biology of the fungus in relation to its survival in nature and its response to the environment. In this aspiration, the biochemist remains the handyman of the biologist. The patterns unfolding in activities of the bacteria and the fungi, and to a lesser degree, in those of the other groups of microorganisms, render it not only desirable but perhaps inevitable that an integrated coverage of the various groups of microorganisms be presented in college courses intended to provide well-rounded training and outlook in microbiology. Possibly one can already detect a trend in that direction. It is significant, perhaps, that nowadays one occasionally sees courses, and even departments, called "microbiology," where once they were named "bacteriology." Instead of the present compartmentalized treatment of these subjects, it would be enormously more advantageous to students and to the progress of research in all areas of microbiology to have a kind of United Nations among microbes; the most effective agency of this organization would be microbial metabolism.

Whereas, a generation ago, the subject of microbial metabolism might have been an amorphous amalgamation of the component specialities, today we have a powerful

orientating force welding them into a discrete pattern; that force is the doctrine of comparative biochemistry, promulgated in 1926 by Kluver, in collaboration with Donker.

A corollary of this doctrine now has become a practical working guide, that is, wherever a unique metabolic character is found, or a metabolic character is found in an unusual degree, its study eventually is bound to have significance transcending the organism originally studied. It becomes, so to speak, a metabolic archetype, a metabolic model. Herein lies the greatest virtue of the microbiologist—he can pick his system. And so, to elucidate reactions of broad biological significance, it has become the rationale to select for study from the infinite variety available some one or a few microorganisms that display the unique intensity.

### *Historical Studies on Fungi*

There is an ample number of such metabolic models in the fungi, and I should like to select a few with which to make special points. Historically, we may start at the very beginning of experimental microbiology, namely, with Pasteur. Having formulated the new concept of fermentation as a mode of respiration of brewer's yeast in the absence of oxygen, Pasteur became convinced of the broad biological significance of this discovery only after showing that various filamentous fungi behaved in the same way as did yeast. In his famous "Études sur la Bière," published in 1872, Pasteur wrote, ". . . the study of varieties of the genus *Mucor*, grown in natural or artificial saccharine liquids, is of great importance to the establishment of the physiological theory of fermentation. . . ." Similarly, a subject of enormous importance nowadays in biochemical genetics and in industrial microbiology is the problem of the profound differences in metabolic behavior

among organisms which are morphologically indistinguishable. The existence in microorganisms of evolutionary divergence of morphology and of metabolic patterns for securing energy is regarded as elementary in modern microbiology. Yet, it was in his studies on the fermentative tendencies of filamentous fungi that Pasteur, more than three quarters of a century ago, made apparently the first definitive observations on the existence of strain specificity, which he called "physiological polymorphism."

Here, too, we may mention the very beginnings of experimental studies on trace-element nutrition, employing the ubiquitous mold *Aspergillus niger* as the test system. To these studies, published in 1869 by J. Raulin, who was Pasteur's first doctoral student, may be traced the elegant modern studies on trace-element function in enzyme action.

Although the record shows that the aforementioned historical studies on fungi, as well as many others which could be cited, stand clear in their anticipation of later developments, it would be poetical to assume that they were directly responsible for the revolutionary developments occurring much later. I prefer to think that, however well documented, the metabolic studies on fungi in the early days made little impact on general biology because they were regarded as unique to that particular group of organisms, the fungi, which were virtually unknown to investigators not specializing in them. Only much later, when the particular metabolic phenomena were rediscovered in plant tissue, in animal tissue, in bacteria, and in yeast, were they exploited, and the significance of the early fungus studies acknowledged through courtesy of hindsight.

One may well wonder what the state of our knowledge in certain areas might have been today had certain phenomena, first recognized in fungi long before their modern culmination, been assiduously exploited when

discovered. Worth mentioning here are a few mold models that many years ago augured developments which today we regard as fundamental throughout the biological kingdom. For example, how much earlier might have been developed our knowledge of transmethylation and one-carbon metabolism in biosynthesis had the remarkable discovery made by the Italian, Gosio, in 1891, in relation to death of humans by arsenic poisoning, been systematically investigated. Gosio showed that in the rooms where poisoning recurred, volatile compounds of arsenic were liberated into the atmosphere by fungi growing on the wallpaper when moisture conditions were suitable. The arsenic compound, Gosio gas, originated from arsenic-containing pigments used for the designs on the wallpaper. Numerous pure cultures of filamentous fungi were proved capable of volatilizing inorganic arsenic, the outstanding organism in this respect being *Scopulariopsis brevicaulis*. Thought originally to be diethylarsine, the garlic-odored Gosio gas later was shown to be trimethylarsine by Challenger (2) and his colleagues at Leeds. The extensive work of Challenger on the methylation of arsenic, as well as selenium and tellurium compounds, has recently culminated in the demonstration (3) that the process is in fact a one-carbon transfer. The methyl group of methionine, and probably of choline and betaine, is donated to the inorganic arsenious acid to produce trimethylarsine, via monomethyl and dimethyl intermediate stages. Radioactive formate carbon also is a precursor of the methyl carbons of trimethylarsine, dimethylsulfide, and dimethylselenide, as shown by the Challenger group. Thereby the evidence was completed that methylation of the metals is indeed a special case of the universal biochemical pattern of one-carbon transfer in which the usual methyl donors transfer the one-carbon group to an unusual methyl acceptor—arsenic. Functionally, however, the arsenic is like the usual methyl acceptors. Fantastic as it seems, the curi-

ous property of *S. brevicaulis*, the arsenic fungus, which it displays to a conspicuous (or should we say, lethal?) degree, exemplifies the thesis presented earlier, to wit: in metabolism, the unusual is not only the outpost, but the signpost of the usual—a metabolic model, as it were.

Earlier studies on the fungi might have led to results in still another area, namely, attainment of our modern conceptual formulation of the mechanism of, and the cyclic nature of, respiration and carbohydrate oxidation. A perspective appraisal reveals that individual strains or species of fungi acting on glucose accumulate, under appropriate conditions, a preponderance of one carboxylic acid, generally accompanied by lesser amounts of other acids. Thus we have citric-acid-accumulating fungi; we have itaconic acid fungi; we have fungi accumulating fumaric acid and its relatives, succinic and malic acids; we have fungi producing oxalic acid, with its acetic and glycolic acid equivalents; we have fungi accumulating ethanol, with its acetic acid equivalent. These distinctive fungus processes were recognized as far back as the 1890's. We know today that all these fungi conduct fundamentally the same type of oxidation of the carbohydrate, that the oxidation is the aerobic respiratory process of the fungi, that with one or two exceptions all these organic acids are formed by all the fungi so far examined, that the unique accumulations represent blockage of the oxidative pathway at different points, and that the process is cyclic—the Krebs tricarboxylic acid cycle.

Thus, for a half-century before the conceptual formulation and the experimental demonstration by Szent-Gyorgi and by Krebs of the role of dicarboxylic and tricarboxylic acids in aerobic respiration, the signposts marking the way were clearly visible, albeit unrecognizable, in the fungi. The same might be said for the so-called gluconic acid shunt, because it was in 1922 that Molliard discovered that certain fungi accumulate distinctive amounts of glu-

conic acid from the oxidation of glucose. Perhaps worthy of note here is the recorded oxidation by fungi of other hexoses, and also pentoses, to the corresponding hexonic and pentonic acids (7, 8). It is not unreasonable to suppose that these may represent intermediate stages analogous to gluconic acid in direct oxidative pathways. Lastly in this particular respect are what appear to be some long-recorded manifestations of current evidence for initial stages in new oxidation pathways. These relate to the demonstration that the hexose molecule may undergo novel changes in the oxidation of certain carbons, without prior rupture of the carbon skeleton. I refer here to the rather substantial yields of glucosone obtained from carbohydrates by Walker (14), using species of *Aspergillus*, and the recent isotopic evidence of Arnstein and Bentley (1) and of Denison and Carson (5) that kojic acid formation by *Aspergillus flavus* represents intramolecular oxidation and dehydration of the glucose molecule, without rupture of the six-carbon chain.

We could turn the spotlight on numerous other neglected or unheeded contributions that might have heralded the great developments made later in other biological systems, but that is like watching the stations go by while riding backward on a train. If we reverse our seating position and exercise good vision, we should be able to spot and evaluate the stations as we approach them, before they recede from our view. The discussion of organic acid accumulation by fungi points up a *modus operandi* which, although habitual with investigators of mold metabolism, appears not to have been widely adopted in metabolic studies with other microorganisms, the bacteria in particular. From studies too numerous to cite in detail, it has become well established that ordinarily a fungus in culture converts the bulk of its substrate to cell material and carbon dioxide. Products of incomplete oxidation, that is, the intermediates in the combustion proc-

ess, generally are found in abundant yield only when the concentration of the substrate exceeds a relatively high minimum level. The best explanation of this appears to be that the presence of excess substrate maintains the formation of intermediate products at a rate faster than they can be transformed; hence they accumulate. Elsewhere, I have described this as "shunt metabolism." The essential result is a higher yield of a particular product per unit of substrate consumed, up to a certain maximum. Although it has been conventional to cultivate fungi in media whose substrate content is 1 to 5 per cent or more, especially where accumulation of intermediates is sought, it is not often that bacteria are cultivated in media containing in excess of 0.5 to 1 per cent of a single carbon source of any type. Especially in aerobic oxidation one might expect, if the bacterium is furnished a high substrate concentration, to find products of incomplete oxidation accumulating. Their nature would afford good clues as to the mode of degradation of the substrate, and thereby expedite the solution of the problem, which otherwise might be difficult of access in media of low substrate concentration. An outstanding illustration of this principle exists in the discovery that numerous species of *Pseudomonas* and *Phytomonas* produce high yields of various hexonic and pentonic acids when the carbohydrate content of the medium is high (9, 11). That this phenomenon is not limited to formation of sugar acids is evidenced by the accumulation of 20 per cent molar yield of  $\alpha$ -ketoglutaric acid from 5 per cent glucose by a strain of *Pseudomonas fluorescens* (10). Undoubtedly the same principle would apply, and awaits exploitation, with respect to intermediate oxidation products of substrates other than sugars. It is already known that various strains of *P. fluorescens*, when furnished adequate ethanol as the substrate, carry out an acetification process indistinguishable from that formerly thought restricted to the genus *Acetobacter* (13).



Although this instruction has been available in the area of mold metabolism for more than a half-century, it should not be inferred that capitalization to the highest degree possible has already been accomplished with the fungi themselves. Nevertheless, I am confident that the principle of high substrate concentration as practiced with fungi will, when properly manipulated, stand with other accepted means of obtaining vital information relative to the pathways of oxidative breakdown of virtually any soluble compound by microorganisms.

Two notes of caution seem desirable, to anticipate results inconsistent with this thesis. First, it should be recognized that the initial organism tested on the particular substrate may not display a useful performance. Whereas the biochemist might conclude that that finished it, the microbiologist simply moves in with a magical bag of tricks peculiar to his profession—an inexhaustible series of different strains and species of microorganisms. The second note relates to satisfying the oxygen demand of organisms developing in media of high substrate content. As methods for achieving this are adequately documented in numerous literature sources, it is only necessary to comment here that the problem is particularly acute in mold cultures because of the tissue-like nature of the growth.

### ***Catabolic Mechanisms of Fungi***

Among living things, by and large, the fundamental differences in metabolism appear to be those concerned with the procurement of energy, that is, the catabolic activities. To put it another way, and excluding autotrophic forms, the oxidative degradation of each of the infinite variety of single organic compounds existing in nature furnishes the energy and building blocks for at least one species of microorganism; otherwise, during the course of geologic time the particular compound would have ac-

cumulated on the earth to an incredible degree. If one assays the participation of fungi in the vast and inexorable decay process which pervades nature, of necessity one becomes convinced that, along with bacteria and actinomycetes, the fungi play a prominent role. Seeking details of the metabolic processes and pathways whereby the multifarious organic compounds of nature are catabolically attacked by various individual fungi, one does, however rather quickly perceive that the available information deals overwhelmingly with one class of compounds, that is, carbohydrates. Compared to the extensive variety of catabolic mechanisms known in the diverse bacteria utilizing manifold compounds, those known in the fungi can hardly be considered numerous. Undoubtedly, the obvious preoccupation with carbohydrate metabolism of fungi is ascribable, in large degree, to the industrial significance of certain carboxylic acids produced from carbohydrates. The disparity between numbers of bacterial and of fungal catabolic pathways points up vast gaps in our knowledge of mold metabolic types, and at once stakes out enormous areas awaiting what surely will be fruitful exploration.

And the approach here may be quite direct, for the basic requirement is simply an array of pure cultures of fungi, each capable of utilizing a single compound as the sole source of carbon or nitrogen or both. The important thing here is the great number of diverse compounds available for this purpose. When this potential metabolic information becomes translated into real information, the present status of mold metabolism will, in retrospect, appear modest indeed.

Testing of existing stock cultures will not, I should judge, be enough to procure the necessary scope of substrate attack; for, notwithstanding the great number of morphologically different fungi available in pure culture today, one might suspect that the conditions of their original isolation from nature undoubtedly have resulted in more homogeneity than heterogeneity of metabolic types.

Development of new and improvement of existing methods will enhance chances of obtaining from nature varieties of fungi that seldom, if ever, see the light of the laboratory. For example, it is customary to facilitate isolation of fungi by acidification of the medium in order to suppress interfering bacteria and actinomycetes. This method works, but it works to obtain only acid-tolerant species. Actually, a considerable portion of the soil fungus population is also suppressed by the elevated hydrogen-ion concentration. A promising approach to this objective is the use at neutral or slightly acid pH values of various chemicals which selectively inhibit nonfungal organisms.

The application of suitable and ingenious enrichment and selective culture techniques with respect to utilization of diverse individual chemical substrates will, I am sure, reveal that, metabolically speaking, the fungi presently are the most underrated of all the microorganisms. Products of new catabolic pathways may have significance for the applied microbiologist, as well as for his academic counterpart. Metabolic properties hitherto unsuspected in fungi are to be had simply by performing appropriate, skillful experiments. Illustrative of the ranges unsuspected in these organisms is the recent report (6) of the rather decided formation of nitrate from reduced nitrogen compounds by numerous soil isolates of *A. flavus*, grown heterotrophically. I may mention also that M. Dworkin in my laboratory has isolated a mold which makes rapid and abundant growth with ethane gas (99 per cent purity) as the sole source of carbon and energy, apparently the first such fungus described as being capable of utilizing a gaseous hydrocarbon.

### *Synthetic Activities of Fungi*

My concentration up to this point on the catabolic aspects of fungi ought not to be interpreted as a purposeful attempt to ignore the synthetic or anabolic activities of

fungi. Impressive advances in this area of mold metabolism have been made with blocked mutants of *Neurospora* and certain other fungi. For the most part, the biosynthetic pathways examined have proved to be of generalized biological significance. Remarkable promise still exists, however, in relation to biosynthesis of innumerable unique compounds. Who would deny the probable generalized significance of metabolic steps elucidated from a study of, for example, the extensive list of hitherto unknown molecules, including aromatics, isolated in significant yields from fungi by Raistrick and his collaborators (12), or of the rather formidable list of antibiotic compounds synthesized by fungi? Or the conversion of ionic halogens to organic halogens, a process shown by Clutterbuck and his collaborators (4) to be virtually universal in the 130-odd fungi tested? These few examples suffice to portray the frontiers of unique biosynthesis presented by the fungi.

For a moment, I should like to return to a point mentioned earlier, namely, the one-sided emphasis placed on metabolism of carbohydrates by fungi. From the standpoint of obtaining synthesis of cell material and commercial products with greatest dispatch, one can appreciate the special interest in the pathways of carbohydrate metabolism. Also directing interest toward carbohydrate metabolism is its manifest significance for animal and human nutrition. As a result of these preoccupations, there is the danger of overlooking the perspective of the mold as a biological entity unto itself, and the manner by which it effects biosynthesis of its protoplasm under the conditions of survival in nature.

The question is: to what extent does carbohydrate metabolism furnish the energy and carbon for growth of a fungus in its natural habitat? Certain considerations of microecology suggest that the opportunity to utilize soluble carbohydrates in nature is a privilege reserved for a very small minority of the vast spectrum of microbiological

entities existing in nature. For example, it is a well documented fact that numerous species of microorganisms are unable to utilize carbohydrate as their sole source of carbon and energy. On the other hand, those organisms which are proved capable of utilizing carbohydrate may not, and indeed probably do not, have the opportunity to exercise that capacity under the conditions which may exist in the soil. Irrespective of the amount or the origin of soluble carbohydrate, the vast majority of the microbial population could well lose out in the competition for it to the fastest growing minority populations in the soil. For example, the bacteria would, in this respect, be formidable antagonists for most fungi, except in acid soils where the bacteria are differentially suppressed.

If we examine further the capabilities of the soil population, we find innumerable soil forms eminently well adapted to utilize various one-, two-, and three-carbon compounds as the sole source of carbon and energy. This point strikes at the heart of a thesis for which ample support might be adduced: the synthesis of protoplasm from one-, two-, and three-carbon split products derived from the dissimilation of larger molecules by fast growing minority populations may well account for a much larger proportion of the gross biosynthetic activity in the soil than hitherto appreciated. And since fungi, as a rule, appear to be metabolically sluggish compared to most bacteria, this ability to survive on metabolic "scraps" may be widespread among, and perhaps characteristic of, many molds and other microbial growths in nature. In any case, I strongly suspect that in microorganisms, pathways to total biosynthesis from one- and two-carbon compounds may yet emerge and prove to be as significant as sugar breakdown in the over-all foundations of biosynthesis in nature. An analogy to photosynthesis will be perceived, especially in respect to intracellular self-regeneration of appropriate acceptors via cyclic mechanisms.

In conclusion, we have seen how unique metabolic features of fungi, long recognized, might have led to major advances in our knowledge of living things in general, for they surely were models of things which came later. We have also scanned the fungus field ahead and, although sketchily, have indicated the existence of innumerable additional metabolic features which, if investigated adequately, are bound to turn out as workable models of much larger replicas in nature.

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## Chapter 7

# *Metabolic Pathways*

By WAYNE W. UMBREIT

The body of knowledge recalled by the words "metabolic pathway" is an important part of microbiology. It is perhaps the most intellectual of the fields of microbiology, possibly the least practical; let us hope that it is not the most obscure.

In the living cell there are varied processes, some of which are dependent upon physical and spatial forces, on tensions, and in a molecular sense, on springs and coils; but the vast majority of living processes are chemical processes, and the living cell, particularly the bacterial cell, is a chemical machine—a chemical mechanism.

The individual reactions that constitute the chemical processes of living tissues, be they human or microbial, have been intensively studied, and a good deal is known about them. No one would assume, however, that with our present knowledge we are capable of describing all of the varied chemistry of the living process. Further, of those reactions whose course is known, the detailed mechanism may be considerably different from what we now suppose. I do not propose to outline detailed reaction

schemes or individual reaction sequences; rather, I shall attempt to draw the necessary inferences and at least the tentative conclusions that I consider are derivable from our present information about metabolic pathways. Although these are always a matter of personal selection, I hope they will be of sufficient significance to provide a reasonable perspective of this area of knowledge.

### ***Enzymes as Surface Catalysts***

In the beginning, I shall take it for granted that the chemistry of the living process occurs at the surface of large protein molecules. Probably no one any longer doubts that enzymes are surface catalysts, that molecules are adsorbed from solution, that the nature of the surface of the enzyme protein is such that particular types of molecules are preferentially adsorbed out of the many available in the cell solution, and that on the enzyme surface these molecules are oriented for reaction. It is further evident that many, if not all, of the reaction processes take place at the molecular surface and because of the orientation. I would assume that this is generally accepted, if only unconsciously, by almost everyone working in the field of metabolism.

This fact of surface catalysis is implicit in all subsequent discussion. But in addition to conducting its chemistry on the surface of protein molecules, the living cell exploits the principle of spatial separation even further by organization into nucleus, mitochondria, and perhaps other cell regions of differing metabolic activity. And even more, the cell possesses at its interface with the environment a membrane endowed with unique surface properties, which have far-reaching and even quite practical consequences. Weighing my words with utmost care, I am prepared to defend the proposition that life does not exist except in cells, and that it is to utilize to the last degree the spatial



properties of matter that cells are necessary, are indeed the vital necessity, for life.

### **Characteristic Metabolic Reactions of Cells**

Within this framework of space relations, and as part of its impact upon the nature of cell reactions, the type of reactions characteristically part of the living pattern can be discerned from the studies of metabolic pathways. There are, to my mind, three such types of reactions which are particularly important, since they are characteristic of the living chemistry. The first is the predominance of group transfer. The second is the organization into reaction sequences of fixed order, some of which are cyclic, others not. The third is the competitive pattern of such reactions or reaction sequences. To the best of my knowledge, these three characteristic metabolic reactions occur in any living cell, and are not unique to the microbe. We shall postpone, for the moment, a consideration of the important and distinct role that microbiology has played in the accumulation of this knowledge until we have considered the knowledge itself, and where possible, its significance for the understanding of the living process.

It seems probable to me that a great deal of the chemistry of the cell is conducted by way of group transfer and exchange. The pattern which I feel is emerging is that there is *de novo* synthesis of relatively few structural types and that from this stage on, the entire structure or critical portions thereof are moved from one combination into another. The now classical cases of group transfer—methyl, acetyl, amino, guanido, and saccharide groups—are but the beginning; and transpeptidation, transamidation, and the exchange of bases by the desoxyribosides are reflections of a basic metabolic mechanism that we may reasonably expect to be employed in even more complex reactions.

This mechanism of group transfer would seem to repre-

sent an enormous advance in economy and efficiency of metabolic reactions. An acetyl group is derivable from a variety of sources; but once an acetyl, its origin is no longer apparent, and the acetyl on its carrier may be in equilibrium with a variety of substances—such as fatty acids, citrate, amino acids, and acetate; then when an acetyl group is needed, it must be much simpler for the organism to draw upon the common supply of acetyl than to synthesize it *de novo* for each separate requirement. It is therefore curious, and I believe significant, that the principle of group transfer occurs in even the smallest and presumably the simplest, in the sense of the most primitive, cells.

The second characteristic pattern of the metabolic pathways as found in living cells is the organization into fixed order reaction sequences. A substance A is transformed to substance B, from here on it must go from B to C, from C to D, etc., until it emerges as substance X or Y or Z. But once it is started along the path, its subsequent fate is determined for the next five or ten or perhaps even twenty transformations. As an example, an acetyl group on coenzyme A has before it an array of pathways, but once it has condensed with oxalacetate to form citrate, its subsequent fate is to a considerable extent determined. What else can happen to citrate but conversion to cis-aconitate, then isocitrate, then oxalosuccinate, then  $\alpha$ -ketoglutarate? Even at  $\alpha$ -ketoglutarate there are only a few alternatives, and beyond, half of the acetyl is already lost.

Such fixed order reaction sequences abound in the living cell. Some are cyclic, some are not, but organization into cycles is not a necessary part of the characteristic sequence. Our present picture is that such paths are tubes or conduits, rigidly confined. They are turnpikes or *Autobahns*, not byways or country lanes. In this respect, the word "pathway" is not exactly suitable, since "pathway" connotes a somewhat wandering way, not set in steel or con-

crete, capable of easy branching and alteration as circumstances require. But so far as I am able to judge, the metabolic pathways of the living cell are rigid and enclosed. The plasticity characteristic of life, and the adaptive abilities, are more a matter of substituting pathways, and competition between them, than a matter of alteration in a fixed order reaction sequence. I admit that this too-rigid mechanistic view is not a very satisfying one, but I am afraid it is correct. In the long view, it is not surprising; after all, the mechanics of muscular movement in our bodies have not changed for at least 10,000 years. Why expect even more fundamental mechanisms, those of chemical transformations, or energy release, to be any more fluid?

These pathways are usually so constructed that they are self-limiting. I suppose the engineer would call these feedback mechanisms. The word itself has no particular meaning to me, and I prefer the term "self-limiting." The Meyerhof-Embden system, for example, requires both adenosine triphosphate (ATP) (at its start) and adenylic acid (beyond the triose phosphate stage), and will stop if all the adenylic is converted into ATP. The same is true for inorganic phosphate.

In addition, there are "cyclic" processes—as occur in the citric acid cycle, the urea cycle, the fatty acid cycle, or the choline cycle—and they are all constructed so that the rate at which the cycle proceeds is dependent not only upon substrate, but upon what one might call built-in controls. The element of control in such self-limiting systems is exerted by a second factor, also self-limiting, namely, series or product inhibition. The clearest example is the fact that oxalacetate is one of the most potent inhibitors of succinoxidase known. In the reaction series succinate to fumarate to malate to oxalacetate, the oxalacetate must be removed or the series will stop. In brief, there are, in the fixed series metabolic pathways, two factors of control:

self-limiting reactions and series inhibition, which require that the cell operate its reaction pathways essentially one molecule at a time. This molecule-at-a-time requirement permits the free play of the third characteristic factor of the living process, that of competition. There are in every cell alternative pathways that may be followed by a given molecule. Whether a given molecule of glucose-6-phosphate is converted to fructose-6-phosphate, to phosphogluconic acid, to glucose-1-phosphate, or to glucose plus phosphate, which step determines its subsequent fate, depends on circumstances within the cell.

That such competition may be the controlling factor in cell growth and development was forcibly impressed upon us in studies on the mode of action of streptomycin. Here the resting cells possessed what appeared to be a typical citric acid cycle, and it was entrance into this cycle that was inhibited by streptomycin. Further study showed that the citric acid cycle was indeed present but that it was not sensitive to streptomycin. Rather, there existed side by side with the citric acid cycle a hitherto unknown reaction sequence, the first step of which was the formation of a seven-carbon phosphorylated intermediate. This alternative sequence apparently started from the same materials, was in competition with the citric acid cycle for these substances, and led eventually, but by different paths, to the same carbon dioxide and water. Along the alternative route, however, there must be something that the cell required, since this alternative pathway was originally recognized only by its inhibition by streptomycin, and since, when it was inhibited, the cell could not grow.

It seems to me, therefore, that the studies of metabolic pathways have told us the following things about the living process in whatever cells we may find it: First, that the reactions occur at and because of specific surfaces. Second, these reactions are characterized by: (a) predominance of group transfer; (b) organization into reaction sequences

of a fixed order; and (c) competition between alternative pathways.

One more factor should be added. Granting that there are reaction sequences of such a nature that the first step of what happens to a substance determines, for a long path thereafter, what must subsequently happen to it; granting that competition between alternative pathways exists, it follows that the competitive sites are not critical at each reaction step but only at points where fixed path reaction sequences cross. That is, one has lines of metabolism, but at points where these lines cross—at the nexus or node, as it were—there the competitive forces exert their far-reaching effect.

One further question may now be asked of Nature: Are the lines or paths of metabolism many or few? Of the thousands of substances acted upon by the living cell, are there thousands of pathways? The experimental answer is: there are few. Not just one, but a few. There are not a thousand paths from glucose to pyruvate, but there is more than one—three, perhaps five, but no more. The main lines of metabolism of carbohydrate, for example, are no more than perhaps ten at the most. The degradation of an array of substances, at first sight utterly dissimilar, eventually proceeds through the same central intermediary metabolites. The shoes, the ships, the sealing wax, degrade, in principle, through one or another of the major pathways, and their breakdown is not different from that of sugar and spice; only the early enzymatic maneuvers have to be slightly different.

With synthesis, it may be otherwise, but we suspect not. In fact, one may propose that synthesis begins at such crossroads of metabolic paths. In short, there is a certain unity of process in all living forms; not identity, but not wholesale diversity. In a sense, life has been fashioned, not from an identical mold but from a mold designed by the same craftsman using the same tools in the same way. It is

as if a potter today made a vase, yesterday a soup bowl, tomorrow a pitcher; the shape varies, the structure alters to serve function more closely, but the same clay has been turned on the same wheel with the same immeasurable skill.

### ***Contributions of Microbiology to Metabolic Studies***

Microbiology has played a unique and significant role in the acquisition of this knowledge of metabolic pathways and will continue to provide the essential portion of the metabolic information. This fact arises because of two characteristics of microbial metabolism. Because these are sometimes not recognized, we feel it of interest to point them out, since they are, in fact, almost the unique contribution of microbiology to the subject of metabolic pathways.

The first point is that microorganisms by their very variety provide the necessary tools for such study. Considering an array of organisms that are distinguished by bacteriological and physiological methods, which is indeed why we know that they are different organisms, metabolic study shows that each is not a separate, unique, metabolic system. Rather, the metabolism of each type of organism is composed, in different proportions, of familiar metabolic pathways; and, of course, of some as yet unknown to us. The relative predominance of each pathway may be altered in a given organism, both by culture conditions and by genetic pattern. There is, among organisms, a certain overlap, but by the selection of extremes for study one may obtain organisms predominantly of one pathway, as compared to others; and thus we may achieve separation of systems, frequently long before their nature is known. Thus it becomes possible to study separate systems and trace predominant metabolic pathways. Additional techniques of such selection—simultaneous adaptation and

genetic selection—need not concern us here. The point is that the microorganisms by their very variety have provided the tools for the study of separate and alternative metabolic pathways, which in higher cells are frequently so mixed and intertwined that naught but confusion is the result of laborious study.

The second unique contribution of microbiology is that in many aspects the microorganism is a simpler pattern than the higher cell. Not only may the investigator select a suitable metabolic material for study from among the microorganisms, but also the nature of this material is more under his control. It is tissue not normally under nervous or hormonal control, as is that of the animal. Its age may be more uniform, the functional relation of the cells more constant. A slice of kidney contains glomerulae, tubules, secreting and absorbing cells differing in function, doubtless different in metabolism; their relative preponderance and activity subject to forces of diet, tension, and hormones, to competition with other organs, to a variety of external matters that we rarely consider. In short, then, the microbial cell is unquestionably more primitive than that of higher animals, in the sense that it has little functional integration with other cells, and that it functions by and large for itself, not for a larger organism of which it may be a specialized, perhaps even sacrificed, part.

Studies of metabolic pathways provide us with the material for judging pattern as well as mechanism. And the microorganisms provide us with the tools whereby pattern may be characteristically determined. It is not too great an extension of our perspective to believe that over the horizon the determinant of pattern will play the more important role.

## Chapter 8

# *Pathways in Biological Nitrogen Fixation*

By PERRY W. WILSON

Modern researches dealing with the chemical and enzymatic mechanisms of the process of biological nitrogen fixation have been thoroughly summarized, reviewed, compared biochemically, and reappraised in recent years (16, 17, 18). This paper is concerned, therefore, not with chemical or enzymatic pathways, but with the research pathways explored by investigators in this field during the last quarter century. An examination of such pathways may be instructive if they lead upward toward greater understanding, as I hope they do, or amusing if they lead toward even more confusion, as some of you may suspect.

The manner in which research in this field has been directed and controlled by the techniques available to the investigator is impressive. In retrospect, it is a little shocking to realize that a case at least can be made for the thesis that we have been almost prisoners of our methods, and



that our successes, if any, reflect only the inevitability of achieving them, once we entered the groove dictated by the techniques. Even worse, these techniques for the most part represent just about the only ones that could have been used, so that the major contribution of the workers in this field has been to provide the hands (or should I say the feet?) to follow the path that led to a fertile area for exploration. Such a point of view implies no criticism of the investigators, for it is recognized that once an area was discovered they exhibited considerable diligence in its cultivation. This paper provides a few examples from the studies directed toward investigation of the chemical and enzymatic pathways that in one way or another illustrate this thesis.

### *Case History I. Excretion of Chemical Intermediates*

It is appropriate that the first example should deal with excretion of chemical intermediates, since the initial experiments that led to this technique were made by Jacob Lipman at the New Jersey Agricultural Experiment Station.

Let us consider the apparatus and methodology available to the plant physiologist in the earlier 1900's. Figure 1 illustrates the basic equipment used by Lipman (7). According to modern standards, it is not impressive; it consists primarily of clay pots in which to grow plants. The development of such plants could be conveniently estimated by merely looking at them, but if the investigator really wanted to be scientific, he could weigh the plants or even determine total nitrogen. But if the apparatus available consists of clay pots, one makes experiments in clay pots. Lipman's experiments included some in which leguminous plants such as peas and vetch were grown with nonleguminous plants such as oats and rye. The white sand used as substrate was supplied with all plant nutrients

except combined nitrogen; the legume, inoculated with the proper bacteria, was grown in the outer glazed pot; the nonlegume, in the inner unglazed one.

Lipman noted that, at times, the nonlegume grew as though it were being supplied with nitrogen from some source and assumed it to be the legume. "Noted" is used advisedly in this connection, because many of his observations were merely that; but, as these observations were confirmed by analyses, Lipman proposed that nitrogenous compounds were *excreted* from the nodules of the legume to its companion. This explanation was too bizarre for the time and was immediately challenged and then forgotten.

In the late twenties, A. I. Virtanen in Finland, using a similar type of crude apparatus, made the same kind of observation and, unaware of Lipman's earlier experiments, described once again this curious phenomenon in mixed cropping. By now, however, improvements in plant physiological techniques had been made, so he quickly abandoned experiments with the crude clay pot and concentrated on investigations using the definitely more sophisticated type of apparatus illustrated in Figure 2. The details of this technique have been described in Virtanen's stimulating account of his experiments (13). Here we need observe only that his apparatus provided him with a method for studying the excretion under "sterile" conditions, that is, in absence of microorganisms other than the root nodule bacteria.

This improvement in technique enabled him to recover sufficient excreted nitrogen to characterize it even by the relatively insensitive methods available at that time. Because these excreted products appeared to be almost exclusively aspartic acid and its decarboxylation product,  $\beta$ -alanine, Virtanen proposed that the mechanism of nitrogen fixation proceeded by way of hydroxylamine, which then entered the amino acid pool via aspartic acid (13). It is unnecessary to note that we challenged this interpreta-

tion (15), or to discuss the amicable outcome of that disagreement. The important points to emphasize here are: (a) the type of experiment that led to the discovery of excretion was implicit in the extremely primitive apparatus a plant physiologist ordinarily worked with even as late as twenty-five years ago; and (b) the exploitation of the discovery by Virtanen and his collaborators through development of a more ingenious technique was a noteworthy triumph not to be overlooked. In comparison with modern methods, the tool they forged may look disarmingly frail for the task assigned, but it was a considerable advance over anything else available at the time.

### *Case History II. Initial Studies on Enzymatic Pathways*

Studies at the University of Wisconsin at this time had taken an entirely different direction both in technique and goal. Impressed with the success of the microrespirometer recently introduced by Warburg and Barcroft for the study of enzyme mechanisms, and following the lead of Meyerhof and Burk in applying this instrument to biological nitrogen fixation, we attempted to study the properties of the enzyme system responsible for nitrogen fixation in red clover. Our version of a "Warburg" apparatus for study of plants is shown in Figure 3. Red clover plants were grown in nine-liter Pyrex bottles on sand provided with the usual plant nutrients except combined nitrogen. The bottles were fitted with appropriate accessories so that the gas atmosphere furnished the plants was controlled. Some plants were inoculated with the proper bacteria and thus depended on nitrogen fixation for their development; control plants were furnished ammonium-N or nitrate-N. During the summer months when the plants grew best, temperature was regulated by keeping the bottles in a wooden "bath" through which water flowed.

Using this equipment, we studied the effect of  $p\text{CO}_2$ ,  $p\text{O}_2$ , and  $p\text{N}_2$  on the rate of nitrogen fixation.

This apparatus was admittedly crude, but it had one virtue—it required considerable volumes of gases, so when a gas was required to fill out a particular mixture being studied,  $\text{H}_2$  was the natural choice because it was the cheapest. This choice of diluent gas was a happy one, for which we cannot claim any credit, as it was forced upon us by the method used. In one trial, the tank of  $\text{H}_2$  became empty just as the experiment was begun; for several days, it was necessarily continued without the use of a diluent gas until a new tank was delivered. Fortunately, this was delayed, and since the response of the plants appeared to be different without the  $\text{H}_2$ , the experiment was completed without diluent gas. The results were unequivocal—the plants fixed nitrogen better in the absence of  $\text{H}_2$ . Through the courtesy of Professor Roebuck in the physics department we were given a tank of helium, which was difficult to obtain at that time. The necessary crucial experiment in which  $\text{H}_2$  and He were compared as the diluent gas demonstrated that  $\text{H}_2$ , far from being an inert gas, definitely inhibited nitrogen fixation in red clover plants. Further studies provided evidence that this inhibition was competitive (15).

Once again, fullest exploitation of this new tool of research necessitated the forging of a sharper instrument; this was accomplished when the finding was extended to fixation of nitrogen by *Azotobacter* (20). Then, studies on the enzyme system employing  $\text{H}_2$  as a specific inhibitor could be made with the more precise microrespirometer technique. Not only has this particular tool been valuable for exploring the enzymatic system responsible for fixation of nitrogen, but also it has led to investigations that have made significant contributions to our knowledge of the enzyme hydrogenase (3, 5, 11). Finally, it has been directly concerned with uncovering several new agents of

fixation—agents hitherto unsuspected of this valuable characteristic (4, 6, 9, 12, 17).

But in leaving this second case history, once again note that the apparatus used was one that was practically dictated by the fashion of the period, and certainly something like it would have been constructed by anyone embarking on the proposed study. But such a method inevitably led to use of hydrogen as an “inert” gas, and once this choice was made, the discovery of its inhibitory action was only a matter of time. Had we originally employed the more sensitive microrespirometer technique, this finding might have been overlooked, as it certainly was by the investigators using that technique at that time (2).

### *Case History III. Chemical Intermediates by Isotopic Tracing*

The year 1939 marked the end of an era in the research on the chemical and enzymatic pathways in biological nitrogen fixation. By this time exploitation with the two major tools that had been employed so assiduously during the past decade had just about reached the point of diminishing returns. At a meeting of the Third Commission of the International Society of Soil Science held at New Brunswick in early September, representatives of the two schools summarized once more the chief findings revealed by application of the two methods (14, 19) and then engaged in a rather pointless and futile argument as to which approach—the “organic chemical” one of Helsinki or the “physical chemical” one of Madison—would be the more powerful for new revelations.

The beginning of World War II within a few days all but stopped research in this field. By the time full-scale experimentation was resumed in the late 1940's, powerful new techniques resulting directly or indirectly from the accelerated research of the war period were available for applica-

tion to biological investigations, including nitrogen fixation. Among these were superior methods for cultivation of microorganisms, especially aerobes, resulting from the researches on antibiotics; superior methods for isolation and identification of organic compounds by use of paper and column chromatography; and, in particular, superior methods for tracer studies because of the availability of labeled molecules with appropriate instruments for their estimation.

Let us look along the research pathway, and with hindsight put in the record knowledge of which at the time we were only dimly aware. The new instruments were of interest not only because they were a powerful and sensitive tool but also because they profoundly affected our thinking about the type of evidence necessary to establish a biological mechanism and resulted in a much closer scrutiny of the foundations of the logic of our experiments (1). They were even to determine the type of research we would do, for obviously with such bright and shiny tools as these, we would tailor our problems to make use of them. So at Madison we dropped our investigations of the enzyme mechanisms and concentrated on biochemical steps in the pathway. Figure 4, illustrating typical apparatus used for such studies, is included as a reminder of how much the instruments we use today are derived from the physical-chemical laboratory rather than the biological. Many of these powerful new techniques have become so commonplace that it is sometimes overlooked that the workers of yesterday were bold enough to challenge nature without them.

Using these recently discovered jinn of the scientific lamp, we compared the biochemistry of the nitrogen fixation process in different agents and obtained several lines of indirect evidence that  $\text{NH}_3$  and not  $\text{NH}_2\text{OH}$  was the end product of the fixation process (16). The ammonia then apparently entered into the amino acid pool by the

conventional reactions, such as reductive assimilation and transamination. But direct evidence for  $\text{NH}_3$  eluded us until just a few years ago when we began the tracer studies on *Clostridium pasteurianum*. Traditionally, this organism is a sluggish fixer and was believed to be both inefficient and slow. But a study of its physiology and the employment of modern methods of culture (for example, shake flasks) demonstrated that fixation by this organism was almost as good as by *Azotobacter* (10).

When both the rate and the extent of fixation by the clostridia had been thus increased, the organism excreted large quantities of ammonia into the medium—it seemed that a medium good enough for a sluggish fixer was deficient for this new intensive mode of life. The organism apparently was fixing nitrogen more rapidly than it could be assimilated and was discarding the excess into the medium (10). This was direct evidence for ammonia as the end product of fixation, provided that the excreted ammonia could be demonstrated to be “juvenile” nitrogen—recently fixed, and not merely breakdown products of the cells. Before the time of isotopic tracing, this would have been a difficult assignment, but now it was readily tested by employing labeled  $\text{N}_2^{15}$ . Since the excreted ammonia-N was considerably higher in its level of tracer than any other compound found, and was followed by substances such as amides, known to be storage compounds for ammonia, it was concluded that this provided the long-sought-for isolation of the key intermediate in biological nitrogen fixation (21).

Our satisfaction with this result was somewhat allayed by the fact that we could not repeat it with *Azotobacter*. This organism would not excrete nitrogenous compounds in spite of all our efforts; the growth of the cell apparently always keeps ahead of its rate of fixation. Somewhat in desperation, the isotopic dilution method was tried—a method in which we did not have much hope, since it re-

quires for success that an external source of nitrogen come into some sort of equilibrium with its opposite number inside the cell or even in an enzyme reaction chain. Often, such equilibration does not occur, so if a negative result is obtained it may not mean much; but, to our surprise, it succeeded (8). *Azotobacter* cells fixing ordinary  $N_2$  were furnished with a small external supply of labeled ammonia; at intervals, aliquots were taken and the level of  $N^{15}$  de-

Table I. Dilution of Exogenous Ammonia by *Azotobacter vinelandii*

Time in Hours	Atom % Excess $N^{15}$ in		
	Total Culture	Cells	Supernate Ammonia *
Experiment I †			
0	5.97	0.05	25.14
1	5.98	0.92	25.33
2	5.88	2.28	20.84
3	5.71	3.98	12.34
Experiment II ‡			
0	—	0.01	0.05
0.5	—	0.03	0.10
2	—	1.51	2.27

\* Reisolated by alkaline distillation from culture supernate before complete utilization occurred.

† 18-hour culture supplied 40  $\mu\text{g/ml}$   $N^{15}$  as ammonium acetate at 0 time (31.9 atom % excess  $N^{15}$ ).

‡ 19-hour culture supplied 20  $\mu\text{g/ml}$   $N^{14}$  as normal ammonium acetate. Gas phase 0.1 atm  $N_2^{15}$  (31.9 atom %  $N^{15}$ ), 0.5 atm  $O_2$ .

terminated. As shown in table I, the label in the external source of nitrogen decreased rapidly and steadily, indicating that it was equilibrating, with  $N^{14}H_4^+$  appearing as a result of fixation. Likewise, when supplied  $N_2^{15}$ , the organism equilibrated fixed  $N^{15}H_4^+$  with an external source of  $N^{14}H_4^+$ . Although not so clear-cut evidence as that obtained with *C. pasteurianum*, it is a welcome addition to the several other types of findings that implicate ammonia as the key end product in nitrogen fixation by *Azotobacter*.



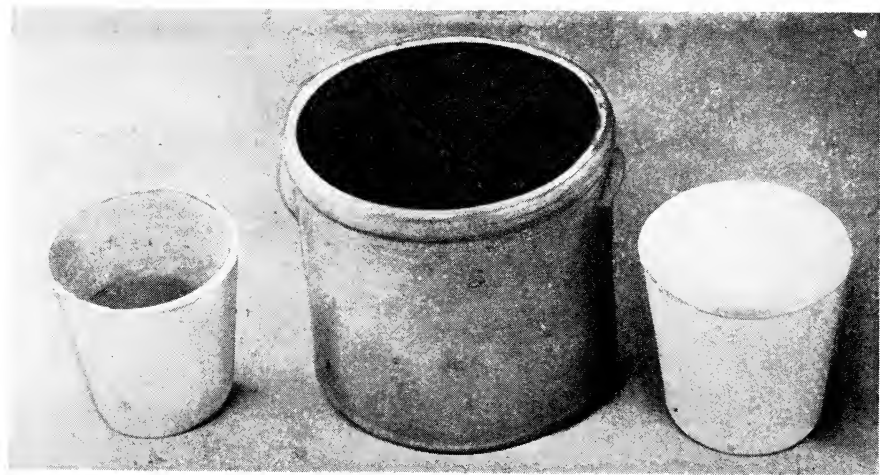


FIGURE 1. Clay pots Used by Lipman in the First Investigation of the Excretion of Nitrogen Compounds by Leguminous Plants.

FIGURE 2. Apparatus Devised by A. I. Virtanen to Study Nitrogen Excretion under Sterile Conditions



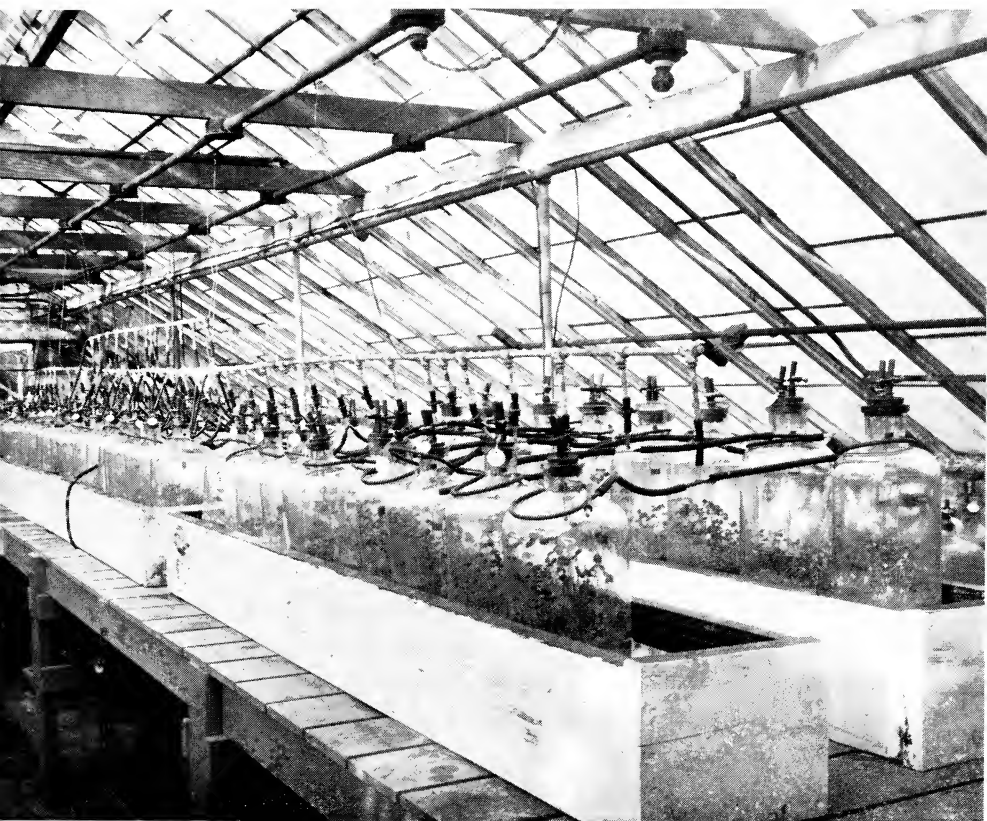


FIGURE 3. Large-Scale "Warburg" Apparatus Developed by Wilson and His Collaborators to Investigate the Effect of Gases on Nitrogen Fixation by Red Clover

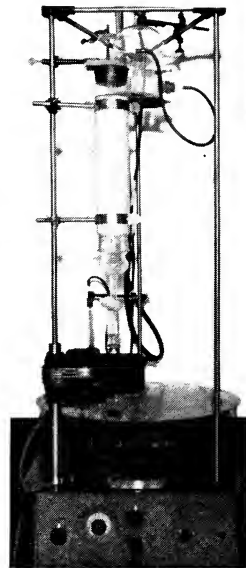
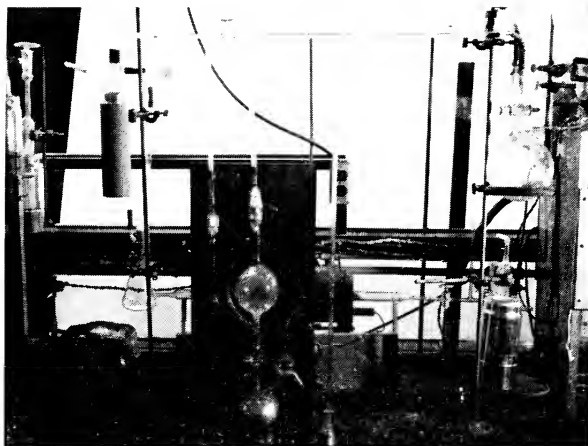
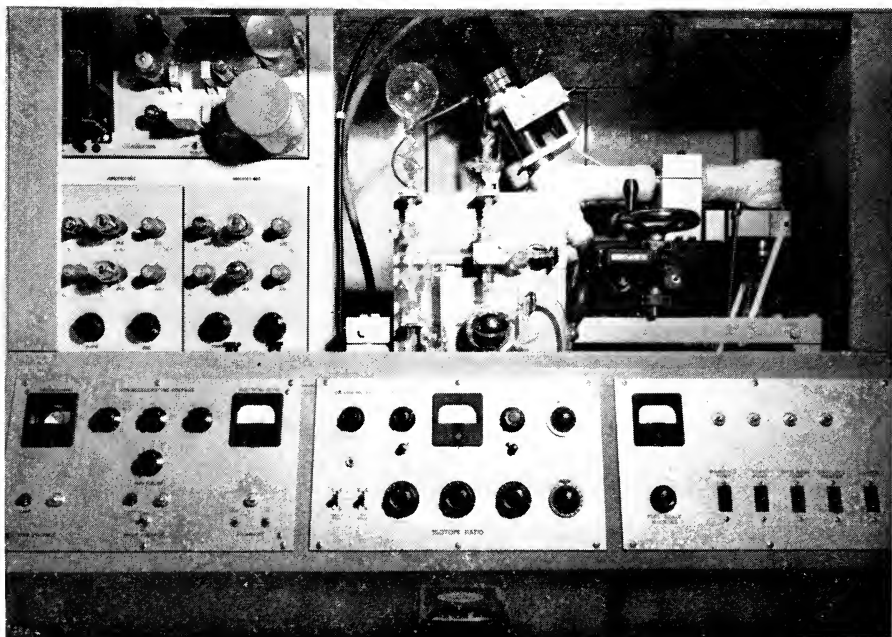


FIGURE 4. Physical-Chemical Apparatus Used in Modern Tracer Studies on the Chemical Intermediates in Nitrogen Fixation

UPPER LEFT: Apparatus for Generating  $N_2^{15}$

UPPER RIGHT: Column Chromatography for Separation of Labeled Compounds

BELOW: Mass Spectrometer for  $N_2^{15}$  Analysis



In leaving this example, once again note that a new method suggested by the advances made in our knowledge of bacterial physiology sooner or later would be applied to the clostridia. Once this was done, the conditions inevitably would be discovered that enabled us to use nature's own method—excretion—for isolating the sought-for intermediate. The extension to *Azotobacter* represents only the introduction of a somewhat more subtle technique to exploit the finding further.

I could cite other case histories from our experience that would illustrate my argument, but I suspect that by this time you are probably recalling some from your own investigations, either in support of or against my thesis. What remains is to draw some suitable conclusion from this survey of the pathways we have used in the past quarter century. Although we might not agree that these roads have led anywhere in particular, I am sure that none would dispute that, at least in the way of complicated apparatus, we have traveled far since Lipman first inserted an unglazed pot inside a glazed one.

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## Chapter 9

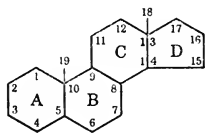
# *Microorganisms and Steroid Transformations*<sup>1</sup>

By DUREY H. PETERSON

Throughout history man has depended upon microorganisms for his existence. The entire life cycle relies in one way or another upon the activity of these miniature chemists. In earliest times man learned to enlist the cooperation of tiny forms of life, as in the preparation of certain beverages, bread, and cheese. As technology advanced, many new and important industries based upon the biochemical activities of microorganisms were developed. These developments culminated recently in such prominent achievements as the production of antibiotics and basic chemicals. Although the application of microbiology to chemical changes has been well recognized in many areas, there was no information available on steroids (formula I) in this respect until 1937, when Mamoli and

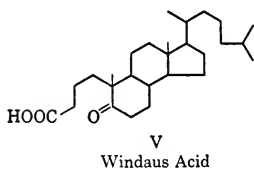
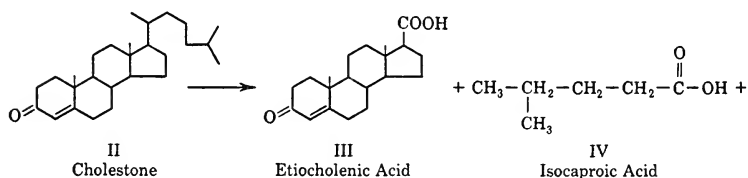
<sup>1</sup> The work reported from the laboratories of the Upjohn Company was conducted in cooperation with S. H. Eppstein, P. D. Meister, H. C. Murray, L. M. Reineke, A. Weintraub, and H. Marian Leigh Osborn and under the direction of D. I. Weisblat and R. H. Levin.

Vercellone (14) first reported the reduction of a nuclear double bond or ketone group, as well as oxidation of a nuclear hydroxyl group. This work was confirmed and ex-



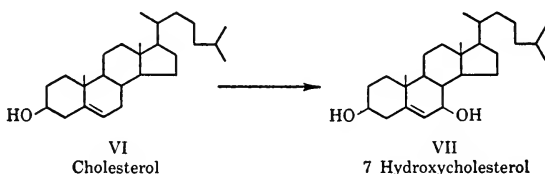
I  
Steroid Nucleus

tended by many other investigators (36). Turfitt (34) showed that cholestenone (II) could be degraded to etiocholenic acid (III) as well as isocaproic acid (IV) and the Windaus keto acid (V) by means of a *Proactinomyces*.



V  
Windaus Acid

Krámlí and Horváth (12) were the first to report microbiological oxygenation of a nuclear carbon atom (position 7 of the cholesterol (VI) molecule), also by use of a *Proactinomyces*, to produce 7-hydroxycholesterol (VII).



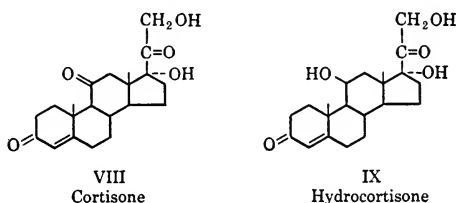
VI  
Cholesterol

VII  
7 Hydroxycholesterol



In 1949, Hench *et al.* (10) observed the dramatic clinical effects of cortisone (VIII), and later hydrocortisone (IX), in the treatment of rheumatoid arthritis. These observations opened the way for one of the most fascinating episodes in the history of medicine and chemistry. The intensive and widespread study that followed was surpassed only by the investigations on penicillin. Although the brilliant work of the organic chemist played an important role in this development, the work reported here on the cortisone and hydrocortisone problem is restricted to the microbiological aspects.

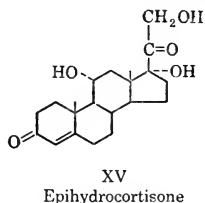
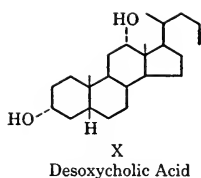
Clinicians were able to show that the 11-oxygen atom in cortisone (VIII) and hydrocortisone (IX) was essential for the biological activity in the treatment of this disease.<sup>2</sup> Unfortunately, chemical introduction of an oxygen atom at position 11 in the steroid nucleus is effected only with great difficulty. In early chemical work, desoxycholic acid (X) from cattle bile was used to make cortisone (VIII),



and it required approximately ten chemical steps to shift the oxygen from position 12 to 11. A total of approximately 37 steps was required to make cortisone from this bile acid.

The major problem, however, was that of introducing oxygen at carbon 11. This necessarily meant that the cost

<sup>2</sup> The structures of cortisone (VIII) and hydrocortisone (IX) are identical except that cortisone contains an 11-keto group, and hydrocortisone an 11 $\beta$ -hydroxyl group. A solid line indicates a  $\beta$ -hydroxyl group lying on a plane facing the reader; a dotted line indicates an  $\alpha$ -hydroxyl group lying behind the molecule. The 11 $\alpha$ -hydroxy epimer epihydrocortisone (XV) is biologically inactive by the rat liver glycogen assay. (5, 27)



of the drug to the patient was prohibitive and cheaper methods for its synthesis were imperative.

The basic structural requirements of steroids necessary for the biological responses observed clinically in rheumatoid arthritis to date are found only in cortisone (VIII) and hydrocortisone (IX) or their derivatives.

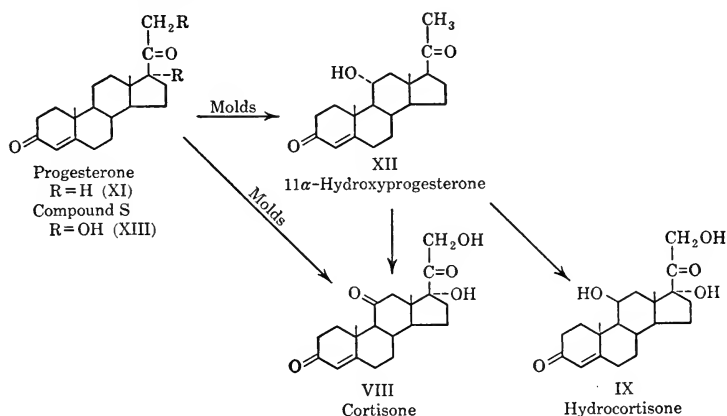
In view of the many complex chemical reactions performed by microorganisms, it was hoped that a microbiological approach might lead to a simpler method. Such a method might introduce oxygen into the critical 11-position of readily available steroids to produce cortisone (VIII) and hydrocortisone (IX) directly or to produce 11-oxygenated intermediates which could then be readily converted to these important compounds (VIII and IX) by chemical methods.

The microbiological oxygenation of steroids at nuclear positions other than 7 was first reported from our laboratories (29) in 1952. That report outlined a one-step method for the introduction of oxygen into the strategic carbon 11 position by fungi of the order *Mucorales*, and more specifically, described the bioconversion of progesterone (XI) to a new compound 11 $\alpha$ -hydroxyprogesterone (XII). Confirmation of this work was obtained by Mancera *et al.* (15) and by Kahnt *et al.* (11). Fried and co-workers (5) independently reported 11-oxygenation of steroids by an *Aspergillus* (Wisconsin strain) (21, 23). Colingsworth *et al.* (1, 7) showed that Reichstein's compound S (XIII) could be converted to hydrocortisone by *Streptomyces fradiae*. Further confirmation of this type of transformation was ob-

tained by Hanson *et al.* (8), employing *Cunninghamella*, and later by Shull *et al.* (33) using a *Curvularia*. Thus, in contrast to the many steps required by the chemist to introduce the important 11-oxygen atom, certain fungi produce 11-oxygenated steroids in a one-step process from readily available steroidal substrates having no special features in the 11 or 12 positions.

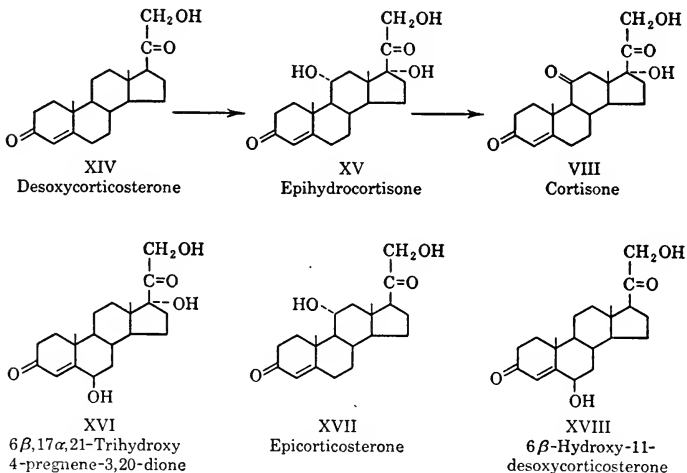
Compound XII (11 $\alpha$ -hydroxyprogesterone) can be readily and economically converted to cortisone (VIII) (15, 26) or hydrocortisone (IX) (13).

Extensive work by our laboratories (3, 21, 30) and by Fried *et al.* (5) showed that many different substrates could be oxygenated not only at carbon 11 but also in other nuclear positions.



Next to the 11-oxygen function, the 17 $\alpha$ -hydroxyl group of the adrenal hormones is the most difficult to introduce chemically, particularly in the presence of a 3-keto- $\Delta^4$ -system. We have recently found that the mold *Cephalothecium* (20) can hydroxylate steroids in this position. This is the first report of 17-hydroxylation of steroids by microorganisms. Other hydroxylating enzymes, 6 $\beta$ - and 11 $\alpha$ -, for example, are also produced by this mold, so that from a single fermentation various combinations of dihydroxyl-

ated compounds are also formed. Thus, for example, desoxycorticosterone (XIV) can be converted directly to epihydrocortisone (XV), a compound which can be readily oxidized to cortisone. Other compounds formed in this bioconversion are  $6\beta,17\alpha,21$ -trihydroxy-4-pregnene-3,20-dione (XVI), Reichstein's compound S (XVII), epicorticosterone (XVII), and  $6\beta$ -hydroxy-11-desoxycorticosterone (XVIII).



Obviously, a combination of chemical and microbiological reactions can be integrated into various synthetic procedures for the preparation of cortisone and hydrocortisone. The final choice as to the best production method depends upon the economics of the starting materials and the efficiency of the processes involved.

### **Chemical Reactions Performed by Microorganisms on Steroids**

Various types of chemical reactions performed by several different molds using various substrates are summarized in the following paragraphs.

OXYGENATION OF STEROIDS BY INTRODUCTION OF A  
HYDROXYL OR KETONE GROUP ON THE NUCLEUS

On the basis of some of the more extensively studied microorganisms, it has been found that hydroxyl groups can be introduced as follows.

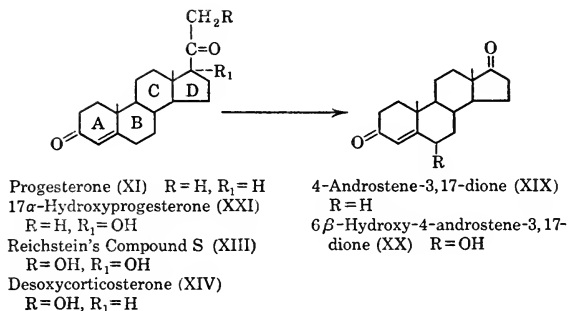
Carbon No.	Orientation	Microorganism
6	$\beta$	<i>Mucorales</i> (2, 19, 21, 27, 28, 30) <i>Aspergillus</i> (5, 21)
7	$\beta$	<i>Mucorales</i> (21) <i>Proactinomyces</i> (12)
8	?	<i>Mucorales</i> (21)
11	$\alpha$ & $\beta$	<i>Mucorales</i> (2, 3, 8, 16-22, 27, 29, 30, 31) <i>Streptomyces</i> (1) <i>Curvularia</i> (33)
14	$\alpha$	<i>Mucorales</i> (16, 21)
15	$\alpha$ & $\beta$	<i>Penicillium</i> (4, 22)
16	$\alpha$	Actinomycete (25)
17	$\alpha$	<i>Cephalothecium</i> (20)

In addition to the foregoing types of compounds, dihydroxylation has been obtained in the following combinations,  $6\beta,11\alpha$ -;  $7\beta,11\alpha$ -;  $6\beta,17\alpha$ -; and  $11\alpha,17\alpha$ -. *Cunninghamella* (8, 21) and *Curvularia* (33) have been shown to introduce an 11-keto as well as an  $11\beta$ -hydroxy group on the steroid nucleus as in cortisone (VIII) when Reichstein's compound S (XIII) is used as the substrate.

RUPTURE OF CARBON TO CARBON LINKAGES

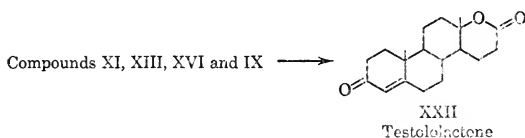
*Cleavage of the Side Chain.* Compounds such as progesterone (XI), desoxycorticosterone (XIV), and Reichstein's compound S (XIII) can be converted by cleavage of the side chain to 4-androstene-3,17-dione (XIX) by certain species of *Gliocladium* (28), *Penicillium* (28), *Aspergillus* (28), and *Fusarium* (35).

The side chain of cholestenone as shown by Turfitt (34) can be cleaved by a *Proactinomyces* to produce etiocholenic acid.



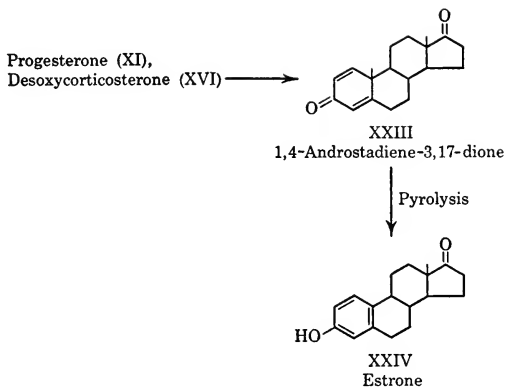
*Cleavage of the Side Chain and Oxygenation of the Nucleus.* With progesterone (XI) *Gliocladium catenulatum* also produces 6 $\beta$ -hydroxy-4-androstene-3,17-dione (XX) (28).

*Cleavage of the Side Chain and Fission of Ring D.* With compounds XI, XIII, XIV, and XXI the side chain is removed by certain fungi of the *Aspergillus* and *Penicillium* groups (28). In these cases ring D is transformed into a lactone to give testolactone (XXII).



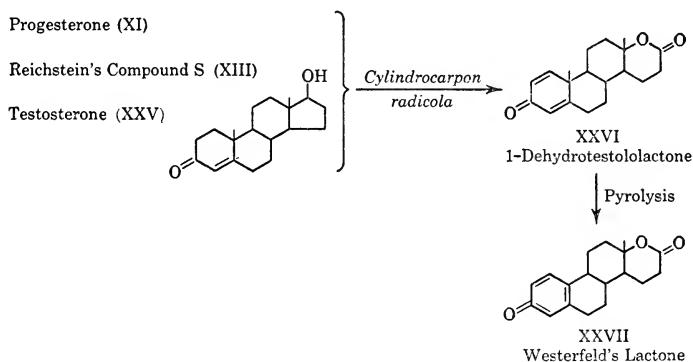
#### DEHYDROGENATION OF RING A AND CLEAVAGE OF CARBON TO CARBON SIDE CHAIN

*Streptomyces lavendulae*, as shown by Fried *et al.* (6), and certain *Fusarium* species, as reported by Vischer and Wettstein (35), are capable of degrading the side chain and dehydrogenating ring A in compounds like progesterone (XI), desoxycorticosterone (XIV), and other steroids to produce 1,4-androstadiene-3,17-dione (XXIII). (This compound can then be easily transformed to estrone [XXIV], an estrogenic hormone, by pyrolysis [Inhoffen reaction].)



#### DEHYDROGENATION OF RING A AND CLEAVAGE OF THE SIDE CHAIN AND RING D

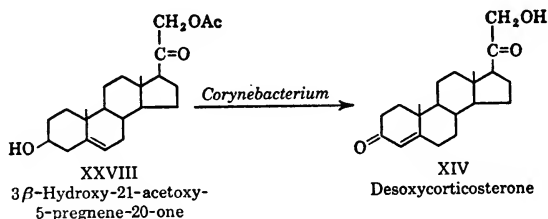
Fried and co-workers (6) have also found that progesterone (XI), Reichstein's compound S (XIII), and testosterone (XXV) can be converted to 1-dehydrotestolactone (XXVI) by *Cylindrocarpon radicola*. (Compound XXVI can then be converted to Westerfeld's lactone (XXVII) by pyrolysis.)



#### OXIDATION OF NUCLEAR HYDROXYL GROUPS

The original work of Mamoli and Vercellone (14) showed that a nuclear hydroxyl group such as is present in  $3\beta$ -

hydroxy-21-acetoxy-5-pregnene-20-one (XXVIII) can be oxidized by *Corynebacterium* to a ketone, desoxycorticosterone (XIV), as shown in the equation.



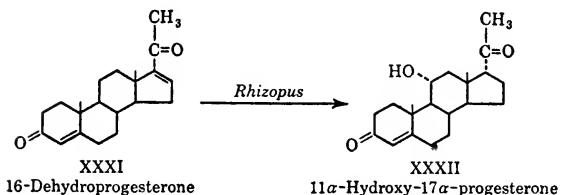
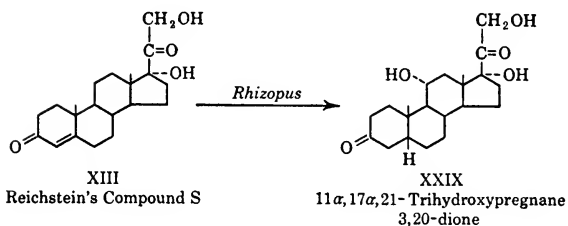
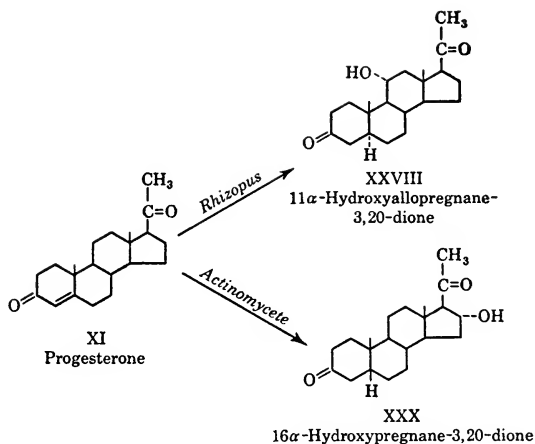
This work has been repeated and extended to other substrates and other microorganisms by several different investigators, including Ercoli, Arnaudi, Molina, Turfitt, Welsch and Huesghem, Zimmerman, Hughes and Schmitt (36), and by Perlman *et al.* (24).

#### REDUCTION REACTIONS

*Ring Double Bonds.* While the bio-oxygenation of compounds XI and XIII by *Rhizopus* produces excellent yields of XII and XV, a reduction of the  $\Delta^4$ -double bond occurs concomitantly to a minor degree. Thus, from progesterone (XI) the 5 $\alpha$ -stereoisomer (XXVIII) is formed, whereas from Reichstein's compound S (XIII) the 5 $\beta$ -isomer (XXIX) is obtained (27, 30). Perlman, Titus, and Fried (25) have shown that a 5 $\beta$ -stereoisomeric derivative (XXX) is formed concomitantly with 16 $\alpha$ -hydroxylation by fermentation of progesterone (XI) with an actinomycete.

The  $\Delta^{16}$ -double bond of 16-dehydropregesterone (XXXI) is specifically reduced in major quantities by *Rhizopus* without altering the  $\Delta^4$ -double bond, and the configuration of the side chain in the resulting compound (XXXII) is the opposite from that occurring naturally (18). An 11 $\alpha$ -hydroxyl group is also introduced as the result of this fermentation. Reduction of the  $\Delta^4$ -double bond by yeast was accomplished as early as 1937 (14).

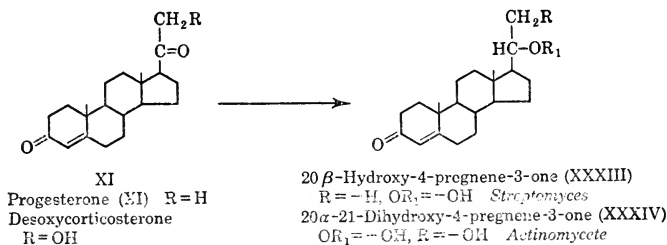




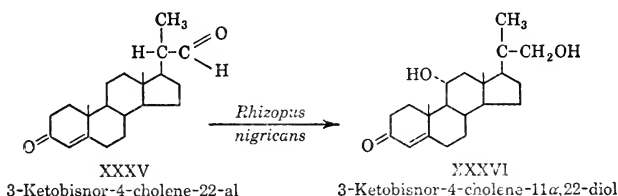
*Reduction of Nuclear Ketone Groups.* Mamoli and Vercellone (14) first showed that nuclear ketone groups could be reduced by fermenting yeast and, by this method, steroids such as androstenedione (XVII) could be converted to testosterone (XXIII). This work has been extended by others (36).

*Reduction of Side-Chain Ketone and Aldehyde Groups.* Fried and co-workers (6) have shown that the 20-ketone of

progesterone (XI) is partly reduced to 20 $\beta$ -hydroxy-4-pregnene-3-one (XXXIII) by *Streptomyces lavendulae*, and work in our laboratories (32) has shown that an actinomycete reduces the 20-ketone group of desoxycorticosterone (XVI) to 20 $\alpha$ ,21-dihydroxy-4-pregnene-3-one (XXXIV).



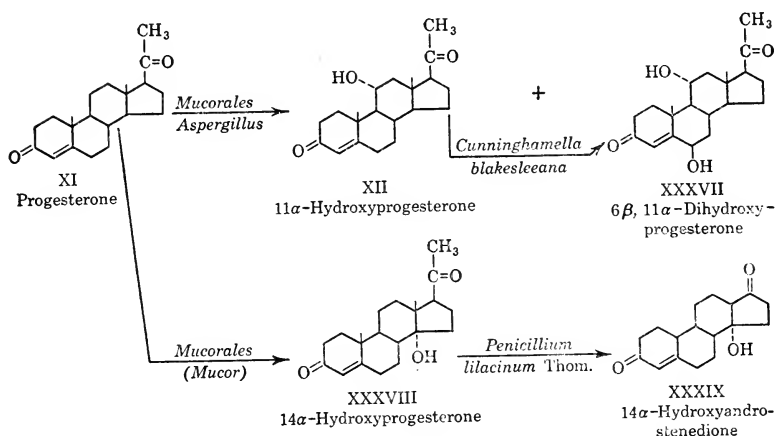
When 3-ketobisnor-4-cholene-22-al (XXXV) is exposed to *Rhizopus nigricans*, the aldehyde group is reduced to a primary alcohol and an 11 $\alpha$ -hydroxyl group is also introduced (17). The resulting compound is 3-ketobisnor-4-cholene-11 $\alpha$ ,22-diol (XXXVI).



Many of the reactions mentioned in this paper are at present extremely difficult to carry out by chemical means, and yet enzymatically these fungi encounter no difficulty in performing the desired reaction.

Several of these microorganisms have been so well charted in regard to their enzyme systems that not only can they be used for preparative purposes, but also they can be employed to determine chemical structure of certain steroids. For example, when progesterone (XI) is converted to 11 $\alpha$ -hydroxyprogesterone (XII), a dihydroxyprogesterone (XXXVII) is formed (13) as a by-product, which was

shown by chemical studies to have a  $6\beta$ -hydroxyl group present. The position of the second hydroxyl group was shown to be  $11\alpha$  when known  $11\alpha$ -hydroxyprogesterone (XII) was incubated with a mold, *Cunninghamella*



*blakesleeana*. The resulting compound was identical in all respects to the dihydroxy-compound isolated from the fermentation of progesterone, thus unequivocally proving the structure as indicated in formula XXXVII.

In a second case, the structure (16) of  $14\alpha$ -hydroxyprogesterone (XXXVIII), obtained by incubation of progesterone with the *Mucorales*, was determined by oxidizing the side chain of compound XXXVIII with *Penicillium lilacinum* Thom. The resulting compound is the known  $14\alpha$ -hydroxy-4-androstene-3,17-dione (XXXIX). The structure proof of this steroid by chemical means would be extremely laborious and difficult.

## Summary

In summary, major emphasis has been placed upon the facile and economical preparation of biologically interesting steroids. Cortisone, hydrocortisone, estrone, and testosterone, compounds which are ordinarily formed by mam-

malian tissue, can now be more readily prepared by the use of certain fungal and bacterial enzymes integrated with a series of chemical transformations. Thus, many complex problems in steroid organic chemistry have been successfully resolved by application of microbiology.

Many challenging problems in this field remain; for example, the mechanism by which the enzymes carry out these transformations must still be unfolded. Hayano (9) recently presented evidence which shows that in the adrenal enzyme system dehydrogenation is not an intermediate step in  $11\beta$ -hydroxylation.

Further applications of microbiology will unquestionably help in the preparation of new steroids that are difficult to synthesize chemically. Undoubtedly, the general field of the microbiological transformation of organic compounds will be widely exploited, and in this respect one can anticipate many new and fascinating developments. Possibilities also lie ahead in the discovery of new types of microbiological reactions which then can be further applied to difficult problems in the field of organic chemistry.

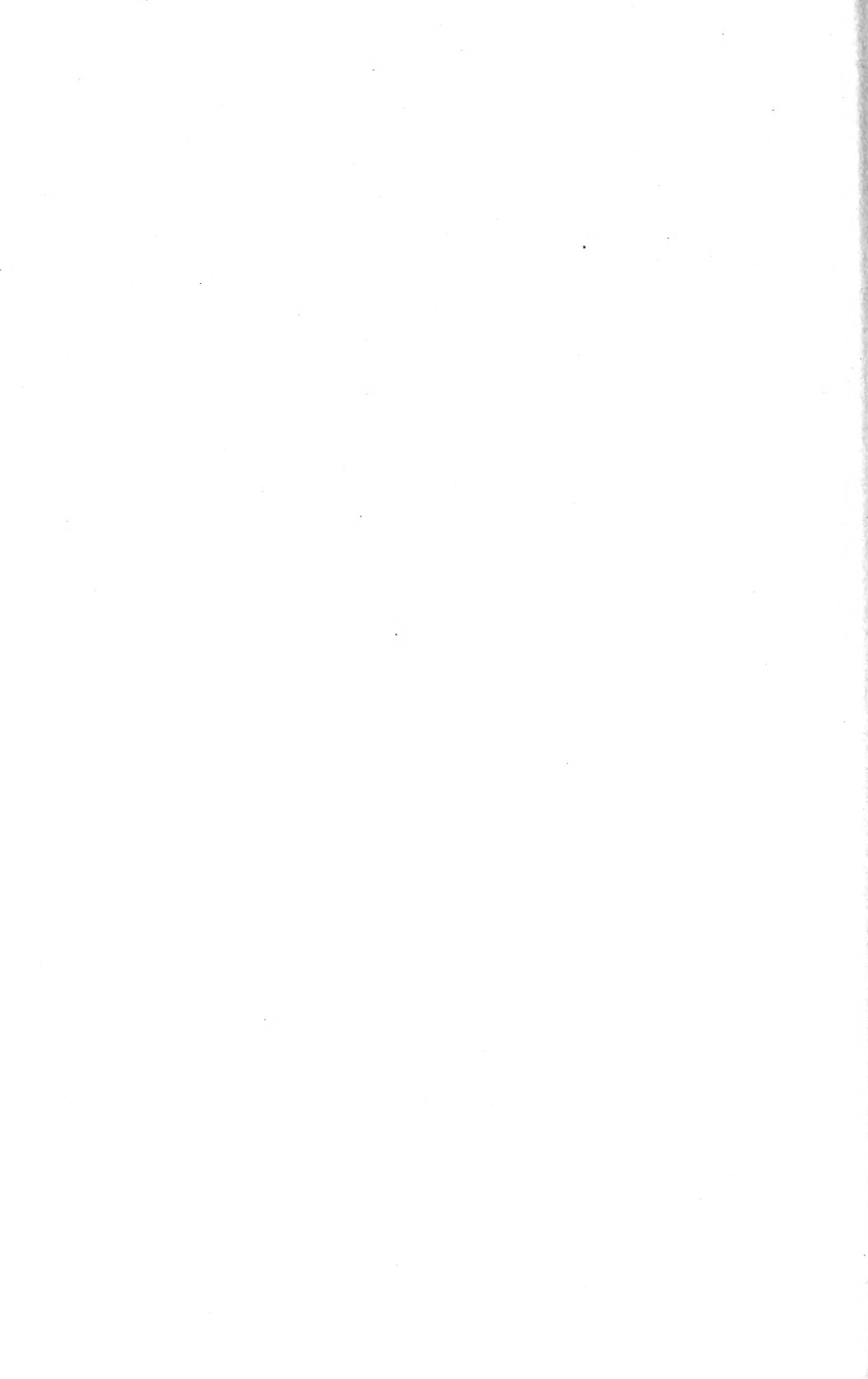
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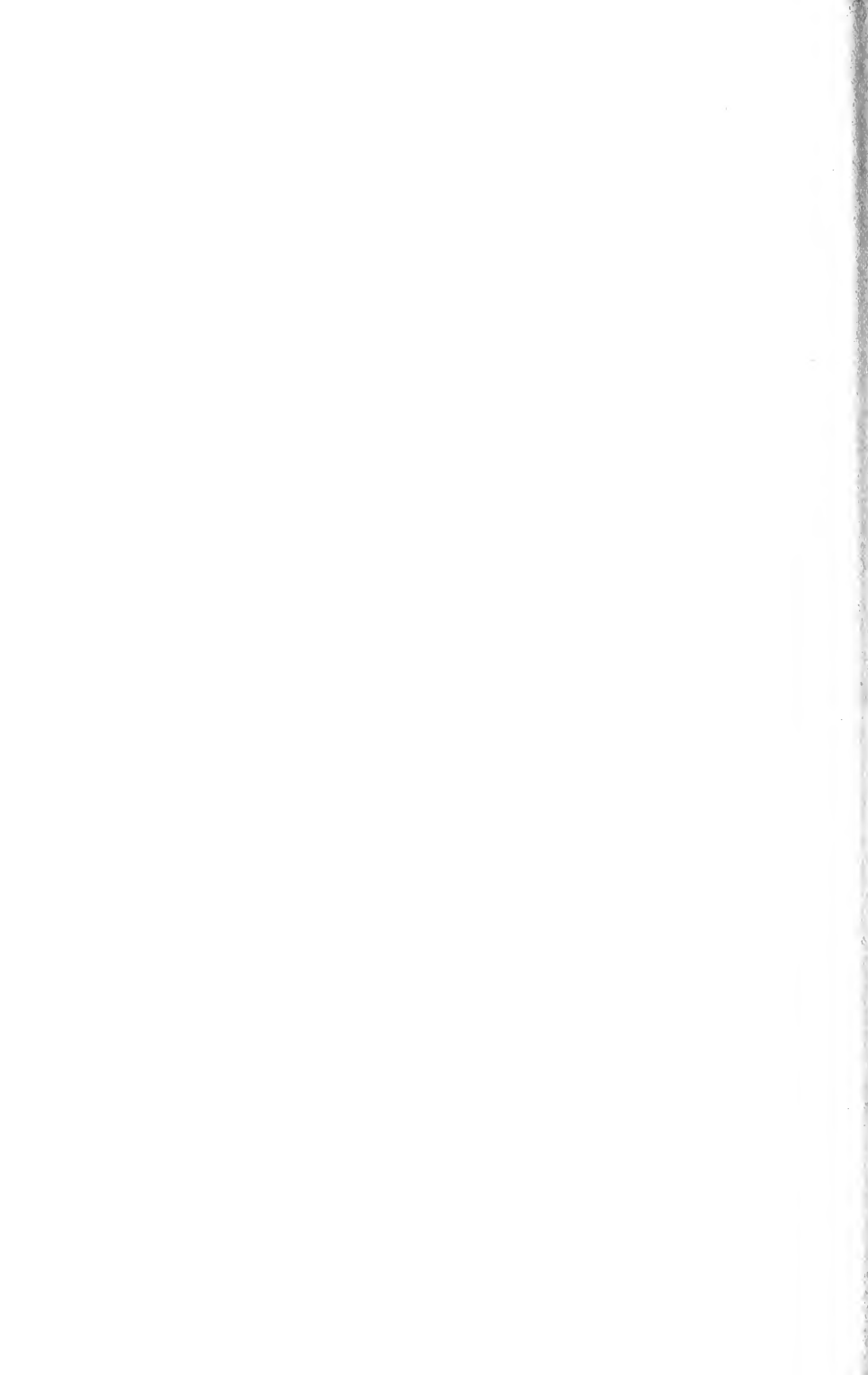
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PART **3**

**MICROORGANISMS AND  
HIGHER FORMS OF LIFE**



## Chapter 10

# *Some Unsolved Problems in Immunology*

By MICHAEL HEIDELBERGER

It is always a grateful task to discuss the unanswered riddles of a science. Not only are these far more numerous than the puzzles that have been solved, but the relatively retarded knowledge of the unsolved mysteries permits one to cast aside the customary restraints of caution, give rein to one's imagination, and fondly suppose that the resulting beams of light will illumine wide corners rather than lose themselves in the murk. Let us, therefore, hopefully select a few outstanding problems in immunology.

Where and how are antibodies formed? There are clues and there are theories, but the latter lack experimental basis.

What are the relationships between chemical constitution and immunological specificity? The pioneering studies of Landsteiner covered the broad range of organic chemistry and posed the problem of the natural antigens in a

more acute form. An impressive beginning on these was made with the sugars by Avery and Goebel, who showed, among many other things, that the mere rotation of a single carbon atom in the chain through  $180^\circ$ , as in carbon 4 on passage from glucose to galactose, was capable of causing a profound change in specificity when these sugars were coupled to proteins. And I shall tell of some more recent experiments with polysaccharides from our own laboratory. But knowledge of the fine structure of the proteins is in its infancy, and so we know nothing of the chemical basis of species or organ specificity or how, if at all, an antibody differs chemically from the corresponding normal globulin.

A major subproblem is concerned with the chemical differences between the blood groups. So far, the hapless victim of improperly matched bloods can tell the difference far more certainly than the immunochemist, who has recognized mainly similarities. But the studies of Morgan and of Kabat are beginning to disclose subtle distinctions among the blood group substances. I shall refer to these.

How does complement "fix" to antigen-antibody systems, and how does complement mediate the lysis of sensitized cells? Is complement or one of its components an enzyme? What is allergy, and how is it related to immunity? I might multiply examples indefinitely. But let us return to the antibodies and consider the gaps in our knowledge of them.

### ***Antibody Formation***

In what organs, or in what cells, are antibodies made? The earlier workers implicated the cells of the reticulo-endothelial system, for it is by these cells that inert particles and bacteria are taken up and removed at least temporarily from the blood stream. Florence Sabin, in 1939, with an antigenic dye prepared in our laboratory gave circum-

stantial evidence that the macrophages were involved, for these cells took up the dye particles and then began shedding surface films at about the time antibodies first appeared. After McMaster's study of typhoid bacilli injected into the lymph nodes, Ehrich, Harris, Dougherty, White, and others experimented widely and demonstrated a connection of lymphocytes with antibody formation, although it was never certain what relation lymphocytic antibodies bore to antibody production as a whole. Finally, a group of Scandinavian workers proposed the plasma cell as the primary synthesizer of antibodies. Following their lead, Astrid Fagraeus devised ingenious experiments that appeared to confirm the important role of these cells. The issue, however, is somewhat beclouded by the failure of cytologists to agree upon a definition of the plasma cell, so that this aspect of the formation of antibodies is still not fully solved.

Neither have we advanced far in our knowledge of the mechanism of the formation of antibodies. According to the theory of Breinl-Haurowitz-Alexander-Mudd-Pauling, an antigen or an active fragment penetrates to the as yet uncertain sites of globulin formation and there, by its presence, so distorts the newly synthesized globulins that they are subsequently able to combine with the homologous antigen, when and if again in contact with it. The Burnet theory, on the other hand, postulates an influence of the antigen on the framework or enzyme systems through which the globulins are built up—a modification which persists as a kind of "training," and so may explain the more rapid secondary response following the reinjection of certain antigens. I have discussed these theories in greater detail elsewhere and have pointed out that the Breinl-Haurowitz theory satisfactorily covers the behavior of antibodies to pneumococcal carbohydrates in man, while the Burnet hypothesis offers advantages in the description of the behavior of antibodies to a protein such as diph-

theria toxin. Both theories represent triumphs of the brilliant imaginations of their authors and are compatible with large bodies of existing data. Decisive experiments in favor of one mechanism or the other, or even an alternative process, are, however, yet to be devised and are urgently needed.

Antibodies may also be considered for a moment as an introduction to the topic of chemical constitution and immunological specificity. Since all efforts have failed to demonstrate differences between normal globulins and antibodies in physical, chemical, or immunological properties other than the capacity to combine with an antigen, Pauling's hypothesis of a difference in folding of the polypeptide chains has gained acceptance by default. New techniques and new experiments are needed in this difficult area.

### ***Specificities of Protein Antigens***

Nor is the question of the specificities of protein antigens in a more satisfactory state. The new methods for the determination of terminal amino acids and their immediate neighbors in the peptide chains of proteins, though laborious, offer hope that the fine structures of the smaller proteins, at least, will eventually be worked out. Until this is done, scarcely anything definitive can be said to relate chemical structure to the specificity of proteins. True, the wide difference in specificities between native and denatured egg albumin, for example, tells us that in the native protein outcroppings of adjacent peptide chains, or clusters of amino acids, probably constitute specific groupings that are altered when their arrangement is dispersed by the unfolding characteristic of denaturation. Paul Maurer has also shown that in the same protein, crystalline chicken egg albumin, about one quarter of the free amino groups may be removed without measurable change in immuno-

logical specificity even by sensitive quantitative techniques, although some physical properties are markedly altered by removal of the basic groups. But these are merely superficial skirmishings and serve only to emphasize the magnitude of the main problem.

### *Immunology of Carbohydrates*

We are, however, not so helpless in dealing with another large chemical group, the carbohydrates. In discussing the immunology of these substances, it was at first necessary to restrict oneself to the bacterial polysaccharides, such as those of pneumococcus. Owing to the vision of Oswald T. Avery, however, it was soon found that various plant gums, notably gum arabic and its product of partial hydrolysis, were capable of reacting with certain pneumococcal antibodies, with the formation of precipitates. The recent discovery that glycogen gives similar precipitates in a number of antipneumococcus sera makes it evident that every carbohydrate is potentially immunologically specific. The immunological reactivity of glycogen is all the more remarkable since this substance is a normal synthetic product of all animals, and hence, according to classical immunological theory, should not take part in immune reactions.

Because the chemistry of the carbohydrates is in a more advanced state than that of the proteins, and because the methods used for the determination of fine structure are not excessively laborious, it has been possible to make several instructive correlations between chemical constitution and immunological specificity among these sugar derivatives.

The first instance in which this could be done followed the demonstration by Goebel and his co-workers that the specific capsular polysaccharide of Type III pneumococcus was a polycellobiuronic acid and that the cross reaction with Type VIII was due to the presence in the Type VIII

polysaccharide of the same cellobiuronic acid. There was, however, no "antigen in common," as the microbiologist is wont to say in reference to cross reactions—only a chemical grouping in common, for the Type VIII specific substance contains glucose in addition. The quantitative aspects of this reaction were carefully studied in our laboratory, and as a result, when cotton was oxidized to a polycellobiuronic acid, it could be predicted that this new substance, soluble cotton, would turn out to be an immunologically specific polysaccharide reactive with Type III and Type VIII antipneumococcus sera. When Stacey isolated a polysaccharide containing multiple cellobiuronic acid groupings from *Rhizobium radicolum*, this substance was also found to react with Type III antiserum. The relation between chemical constitution and the immunological reactivity of the Type III-Type VIII pneumococcus group has therefore been fairly well cleared up as far as the common component, cellobiuronic acid, is concerned. The precise function and points of attachment of the additional glucose radicals in the Type VIII substance remain unresolved.

Another immunologically cross reactive group of wide occurrence might be termed the polyglucose group. Not only do the dextrans formed by various *Leuconostoc* species give cross reactions in a number of antipneumococcus and antityphoid antisera, as shown many years ago by Zozaya and Neill and Hehre, but these substances have been found by Kabat to be antigenic in man. Depending upon the synthesizing strain, dextrans contain glucose in  $\alpha$ -1,6 linkage, with varying proportions of 1,4 linkages and, to a smaller extent, 1,3 links. Some of the synthetic polyglucoses show a serological reactivity in antipneumococcal sera much like that of the dextrans, which is not surprising since Pacsu has found that the synthetic products also contain 1,6 and 1,4 linkages.

A chemical clue to the nature of polyglucose specificities was supplied by Stacey's report that all of the 30 per cent



of glucose in the specific polysaccharide of Type II pneumococcus was in the form of 1,4,6-branch points. This not only accounted for the cross reactions of the polyglucoses in Type II antiserum, but also led to our discovery of the serological activity of glycogen and amylopectin, both of which contain 1,4,6-branch points of glucose. But the cross reactivities of the polyglucoses in Types VII, IX, XII, XX, and XXII antipneumococcal sera remain unexplained, since we do not even know the component sugars in the specific polysaccharides of these pneumococcal types, let alone their linkages. Knowledge of the reaction in Type XVIII antiserum is not quite so limited, for Markowitz and I have shown that the specific polysaccharide of this pneumococcal type consists mainly of glucose, with smaller proportions of rhamnose and secondarily bound phosphate. Most likely, 1,4,6 linkages are not present on any glucose units of the Type XVIII polysaccharide, since there is no cross reaction in either direction with Type II, in spite of the occurrence of rhamnose in both polysaccharides. It is therefore evident that the chemical nature of the polyglucose specificities cannot be fully understood until the component sugars and fine structures of the specific polysaccharides of more of the cross-reacting pneumococcal types are elucidated—a formidable but by no means insuperable task.

Another far-flung immunological specificity which is being unraveled is that of the polygalactoses, and among these I include not only the galactans, but the already mentioned plant gums, since most of these contain galactose in their repeating units. Thus far, the specific polysaccharide of only one pneumococcal type, XIV, has shown this property. Goebel, Beeson, and Hoagland found it to consist of three molecules of galactose to one of N-acetylglucosamine, but did not establish any of the linkages. It was therefore not too farfetched, when Wolfrom and his collaborators isolated a galactan from the heparin mother

liquors of beef lung, to beg some of the material and test it with Type XIV antipneumococcus serum. Nearly one third of the antibody was precipitated. The Ohio workers had shown that galactose was present in the lung galactan in three glycosidically linked forms: otherwise unsubstituted, singly substituted, and doubly substituted. The unsubstituted end groups are glycosidically linked to the 3 or 6 positions of other galactose radicals, and all linkages are 1,3, or 1,6, or 1,3,6. This does not help much, since there are also three galactoses in the repeating unit of the Type XIV polysaccharide, and their linkages are unknown. A clue, however, was furnished by tamarind seed polysaccharide, a commercial thickener of jellies, from India. This carbohydrate consists of a main chain of xylose and glucose units, two thirds of the latter branched at the 1,4,6 positions, making the substance reactive with Type II antiserum. Attached glycosidically to the main chain are single, otherwise unsubstituted galactose units, and these suffice to precipitate a portion of the antibodies in Type XIV antiserum. Moreover, such galactose end groups are the only structures in common with the lung galactan, and since both polysaccharides precipitate Type XIV antiserum, it may be predicted with confidence that not only will all carbohydrates containing unsubstituted galactose units precipitate this antiserum, but that one, at least, of the three galactose radicals in the Type XIV polysaccharide will also be found to occur as an unsubstituted end group. The former prediction has been verified with karaya gum and gum arabic, and it is hoped to test the latter prediction chemically in the near future. Here again, then, part of the problem has been solved, but much remains to be done.

### ***Constitution and Specificity of Blood Group Substances***

The blood group substances present additional puzzles in the relation between constitution and specificity. A most striking finding by all of the more recent workers in this area was the great similarity of the A, B, and O substances, in spite of the dire consequences of the transfusion of mismatched bloods. All three substances were found to consist of galactose, fucose, N-acetylglucosamine, and N-acetylgalactosamine, together with a residue of amino acids. The cross reactivity of the blood group substances in Type XIV antipneumococcus serum has long been known and has been quantitatively studied by Kabat, but it is still not known whether this reactivity is due to the multiple recurrence of certain galactose linkages or whether it is caused by the N-acetylglucosamine portions. Possibly both types of residues function. Leskowitz and Kabat recently devised a new method for the separation and quantitative estimation of glucosamine and galactosamine, and with its aid have shown that hog and human blood group A substances gave glucosamine to galactosamine ratios averaging 1.5, hog and human O(H) 2.3 and 2.6, and human B 2.8. It is thus evident that chemical differences among the blood group substances are beginning to emerge, and the outlook along these lines appears hopeful.

### ***Nature of Complement***

We now come to complement, which remains in large part an enigma despite its importance as an auxiliary in immunity and its enormous use in diagnostic tests carried out daily all over the world. True, our own experiments showed that complement could be weighed, and so disposed of the widely held view that complement was merely a colloidal state of fresh serum proteins. These studies also

led, with Weil and Treffers, to a theory of complement fixation which has not yet been refuted. But we are only beginning to learn how complement mediates the lysis of cells that have taken up antibody. Manfred Mayer has made a good case for the concept that the antibody is the enzyme and that complement merely supplies cofactors or energy. This view is directly opposed to the usual one that complement or one or more of its components exerts enzymatic action on the cell-antibody complex. Progress is, however, being made on the mechanics and kinetics of lysis with the aid of new methods devised by Mayer and his group. These workers have also shown that the initial step of fixation of a portion of complement requires calcium ion and that this is followed by a second prelytic stage of complementary activity requiring magnesium ion. Only then can lysis ensue. In our laboratory, Plescia is working out the thermodynamics of immune lysis, is studying the long-known concentration effect and the range in which lysis is independent of this effect, and has simplified the analytical problem so that large numbers of accurate tests and readings may be made by a single worker. Both approaches appear to be converging toward similar views of the mechanism of immune lysis, but much patient effort and time will still be required before a detailed picture of the complex events can be obtained. Perhaps the final solution may even be delayed until methods for the fractionation of closely related proteins are so improved that complement and its four recognized components can be isolated in a state of purity.

### *Relation of Allergy to Immunity*

As for allergy, we are all familiar with its distressing effects in man and domestic animals, but little is known of the mechanisms of these effects. Is allergy a process running parallel with immunity; is it an imperfect immunity in the

sense of an immunity arrested at too early a stage; or is it an imperfect immunity in the sense of a deviation of the immune process so that abnormal antibodies are formed capable of attachment to certain cells or tissues and there causing damage when antigen appears? The beginnings of an answer may perhaps lie in Mary Loveless's finding of the two separate antibodies involved in the allergic state: the sensitizing antibodies, occurring mainly in the  $\beta$ -globulin fraction of serum, and the neutralizing antibodies, which are found mainly in the  $\gamma$ -globulin portion. And although any protein is potentially an allergen, what is the reason for the extreme potency of some of the large polypeptides such as those found in cottonseed and the castor bean? This would seem an excellent problem for the chemists who occupy themselves with the sequences of amino acids. Moreover, the most active pollen fractions contain carbohydrates, and it is not known whether these are themselves active or are merely adventitious impurities of the actual allergen.

### *What of the Future?*

This brief recital by no means exhausts the list of unsolved problems. I have mentioned enough of these continuing riddles of immunology to indicate that some of them, at least, are almost daily becoming less and less obscure, and that modern quantitative immunochemical methods are supplying, and will continue to furnish, powerful tools for their ultimate solution.

Chapter **11**

*The Inhibition of Virus  
Reproduction by Chemical  
Substances*

By FRANK L. HORSFALL, JR.

When a virus particle and a cell come together, events may begin which lead to the production of more virus particles. If this occurs, it is said that the virus particle is infective, the cell is susceptible, and virus reproduction has taken place. The concepts of infectivity and susceptibility are inseparable; either is dependent on the other. The capacity of the virus to infect cannot be demonstrated without a susceptible cell, while the capacity of the cell to support reproduction is determinable only with an infective virus particle.

If reproduction of the virus leads to abnormalities in cell function, the virus is considered to be pathogenic. When the number of abnormal cells becomes sufficiently large, changes in tissue function may occur. Only when these exceed a certain threshold level can disease be recognized.

Virus disease is dependent on virus reproduction in the sense that, in the absence of reproduction, disease does not develop. Reproduction of a virus does not always lead to disease, however, and some viruses multiply extensively in certain tissues without causing demonstrable abnormalities. There is some evidence that the relationship between the extent of reproduction and the amount of disease is quantitative. With influenza virus or pneumonia virus infections of the respiratory tract in mice, it is feasible to compute how much pneumonia will develop by direct calculation from the concentration of virus in the lung and the time after inoculation (22, 29).

If this relationship has wide validity, a possible means for the control of virus disease opens before us. In theory, inhibition of virus reproduction might be expected to result in less extensive disease. This conjecture has not yet been tested adequately. But the evidence so far obtained with one respiratory virus infection in mice, that is, pneumonia virus of mice, is in accord with the idea. When a chemical substance, in this case a bacterial polysaccharide, is introduced into the respiratory tract, the greater the degree of inhibition in virus reproduction, the smaller is the amount of pneumonia that develops (23). Moreover, the quantitative relationship between virus concentration, extent of lung lesion, and time is identical with that found in the uninhibited virus infection (29). The amount of pneumonia that will develop can be computed from the other two variables.

At first glance, such a result appears to be similar in many features to that found when an antimicrobial agent is employed in a bacterial infection. Bacterial growth is retarded or stopped, and the disease is modified. In the example just described there is, however, a distinguishing feature that may be important. The substance used to inhibit virus reproduction has no effect upon extracellular virus particles and does not prevent their adsorption by

susceptible cells (23, 30). The available evidence indicates that the compound inhibits the intracellular reproductive process. But there is as yet no indication of the means by which this is accomplished. To inhibit reproduction after a virus, or part of it, has entered a cell is not readily achieved. This objective has been realized with chemical substances in only a few instances, but these seem of sufficient importance to warrant further work in this field.

### *Mechanism of Virus Reproduction*

Inhibition of virus reproduction by chemical substances might be achieved more readily if the mechanism were fully understood. Because this phenomenon is an intracellular process inseparable from the life of the cell, it has not been possible to distinguish clearly between the role of the virus and that of the cell. With bacteriophages, large strides have been made recently, and it now appears that the reproductive process involves a number of discrete steps. Current theory holds that phage particles do not enter bacterial cells as intact entities; that their nucleic acids and genetic determinants do gain entry; that certain components of the new particles are produced separately; and that after assembly into mature particles these leave the cell at the time of lysis (25, 26, 31, 40). This complex process can then be repeated in series.

With animal virus reproduction the present situation is far less advanced. There is no good answer to the simple question: do animal viruses multiply themselves, or are they reproduced by the infected cell? Quantitative and kinetic studies, as well as investigations with the electron microscope, have not excluded the possibility that the multiplication of animal viruses may be formally similar to that of microbial species which multiply intracellularly. The rate of appearance of new virus particles after an appropriate latent or lag period appears to be logarithmic in



all cases adequately studied (22, 28, 29), as with bacteria and phages. Development of required precursor materials or of incomplete or immature virus particles in advance of infective particles has not been decisively demonstrated (28, 34), as with phage. Recent studies with influenza virus indicate that when cells are not overloaded by very large inocula, only mature infective particles are produced during reproduction (28).

Both the rate of reproduction and the amount of virus produced are related to oxidative metabolism of the host tissue, and if this is affected, virus multiplication is diminished correspondingly (3, 16). Apparently, depression of almost any metabolic function of the cell, however achieved, results in a depression in virus reproduction. Although evidence of this kind indicates that the virus requires the presence of an actively metabolizing cellular environment, it does not point to any particular biosynthetic process as necessary for reproduction. Moreover, such evidence does not prove that the virus is multiplied by the cell.

If animal virus particles do not enter the cell as intact particles, as is thought to be the case with bacteriophages (25, 26, 31, 40), and only their nucleic acids and genetic machinery are required to set the stage for intracellular reproduction, it is apparent that the process is widely different from the multiplication of other microbial species. The indications that the process is different are based on very thin evidence with animal viruses. The evidence most commonly invoked is the apparent disappearance of most of the infective particles shortly after inoculation (21, 24). To explain this phenomenon on a simpler premise, it is necessary to assume merely that the forces which hold an intact virus particle and intracellular components together are considerably greater than those between the free virus and the cell surface. Indications that there are strong forces binding some animal viruses to cell fragments have been

obtained with pneumonia virus of mice (11, 12). It may be recalled that the only means to demonstrate an infective particle is an intact susceptible cell, and that the virus must be free to collide with and remain at the surface of the cell before its presence can be detected. A decisive demonstration that an animal virus particle does or does not enter the cell intact would provide valuable evidence on this problem and give a better indication of the applicability of phage theory to this field.

This issue is of more than theoretical interest and may affect the usefulness of efforts to control virus diseases through chemical inhibition of virus reproduction. Some important information bearing on this point has been obtained with bacteriophages. An acridine compound, proflavine, is strikingly effective in inhibiting the reproduction of phage T<sub>2</sub> (17, 18). The substance affects late stages in the intracellular reproductive process and prevents the development of new infective particles. Thus it might be thought of as an active antiviral compound with chemotherapeutic potentialities for infected bacteria. Unfortunately, however, use of this substance provides no advantage for the infected host cell, and although the full maturation of new virus particles is inhibited, the bacterial cell dies at the same time as do control infected cells (18). It may be significant that proflavine does not inhibit all of the processes associated with phage reproduction. Both new phage protein and nucleic acid are produced, but they are assembled only into tailless phage heads that are unable to infect other bacteria (13). The action of proflavine is not restricted, however, to an effect on the very last stages of the maturation process. There is evidence that neither the new phage protein nor the new phage nucleic acid produced in its presence corresponds precisely with that found in mature phage particles (13). Thus, it appears that although a widespread effect on a number of biosynthetic processes is induced by the substance, this is not

sufficient to protect the host cell from destruction.

Findings such as these raise the possibility that inhibition of an earlier stage of the reproductive process might have a more beneficial result. There are indications that this is the case. Addition of 5-methyl tryptophane early in the latent period prevents phage reproduction, and, in addition, lysis of the infected host cell does not occur (8). Tryptophane reverses this inhibition, and reproduction recommences at the stage at which it was interrupted by the analogue (10). Similarly, chloramphenicol, if added early in the latent period, prevents both virus reproduction and host cell lysis (5). But neither compound, if added during the latter part of the latent period, prevents destruction of the bacterial cell (5, 8). Once the reproductive process has proceeded far enough to yield a few new intracellular virus particles, the fate of the infected host cell appears sealed. Inhibition of further reproduction does not benefit the cell, and although the yield of virus particles is held to a very small fraction, lysis occurs. Moreover, neither 5-methyl tryptophane nor chloramphenicol leaves the metabolism of the host cell unaffected. Both cause reductions in protein synthesis (5).

If the reproduction of animal viruses is not identical with that of phage, there is a possibility that the results obtained with the phage inhibitor systems may have little bearing on animal virus inhibitor systems. There are a number of indications that animal viruses are markedly different from phages. As an example, no animal virus has been shown to possess a tailed structure. This appendage appears to be a constant feature of phage morphology (41) and is considered to provide the physical means for the introduction of phage nucleic acid into the bacterial cell (26). As was indicated earlier, there is no incontestable evidence that animal virus particles are disrupted or fragmented on penetrating susceptible host cells. With phage, such disruption, demonstrated in the case of  $T_2$  (26), is

one of the major premises on which the current theory of phage reproduction is erected.

The chemical composition of animal viruses has not yet been adequately established, partly because of difficulties in purification. It is too early to conclude that such viruses are composed wholly of protein and nucleic acid or that their nucleic acids are of the desoxyribose type, as in the case with phage (9). Recent studies with the electron microscope on ultra-thin sections of infected cells raise the possibility that even relatively small viruses, such as influenza and herpes simplex, may have surface membranes and a central morphological structure analogous to a nucleus (33). Moreover, animal viruses do not usually cause death and lysis of the host cells they infect in nature. Their reproduction is followed by a trickle or leakage of new virus particles (7, 14), not by the lysis and bursting that typically result from the reproduction of phage in a bacterial cell. In a number of instances, as with mumps virus in the chick embryo, extensive multiplication does not result in any demonstrable host cell abnormality. Thus, whatever the mechanism of animal virus reproduction may be, it seems apparent that the effects of the process on the host cell are generally very different from those caused by phage multiplication.

### *Inhibition of Intracellular Reproduction*

Can these dissimilarities be turned to account and lead to effective means for the control of diseases induced by animal viruses? Before this question can be answered, a number of related problems probably will need more study. It is clear now that it is possible to inhibit the intracellular reproduction of certain animal viruses through application of some chemical substances (27). Compounds of high molecular weight and uncertain structure, like polysaccharides derived from the capsules of *Klebsiella pneu-*

*moniae*, are active as inhibitors of some small viruses, for example, pneumonia virus of mice (30), though not for other viruses in the same host species. Compounds of low molecular weight and precisely known structure, like certain chloro-substituted N-glycosides of benzimidazoles, are potent inhibitors of influenza virus reproduction in a tissue culture system and also are active in the chick embryo and in the mouse (38). Numerous other compounds, including analogues of amino acids (2, 6), purine analogues (19), and certain inhibitors of cell metabolic systems (1, 4, 32), have been reported to have inhibitory activity (15). Only certain substances that appear to cause inhibition of the intracellular process of virus reproduction are considered in this communication. Compounds that inactivate extracellular virus particles, prevent their adsorption to susceptible cells, or affect the responses of tissues without affecting reproduction are outside the scope of this discussion.

The 5,6-dichloro derivate of benzimidazole ribofurano-side provides an interesting example of inhibitory activity with an animal virus (38). This compound, which is related in structure both to a moiety of vitamin B<sub>12</sub> and to adenosine, inhibits an early stage in the intracellular reproduction of influenza viruses. It has no effect upon extracellular virus particles and does not diminish adsorption of the virus by host cells. When the substance is added after penetration of the host cells has occurred, the yield of new virus particles is diminished. The extent of the inhibition is inversely related to the time elapsed before the compound is added. Each of the biologically measurable components that result from the multiplication of influenza virus appears in decreased concentration. Infective virus particles, hemagglutinating particles, and even soluble complement-fixing antigen develop in diminished amount (39). There is as yet no decisive evidence that any material with virus specificity increases more than another in the presence of this compound.

At inhibitory concentration, the compound has no effect on the oxidative metabolism of the host tissue and only a slight effect on cellular proliferation in tissue culture (38). These findings may be taken as indications of some selectivity in action. But the nature of the biosynthetic process affected by the compound has not yet been identified, and efforts to block the inhibitory effect with a variety of possible metabolites, including vitamin B<sub>12</sub> or its corresponding riboside moiety, as well as various purines and pyrimidines, have been unsuccessful.

A simpler compound, but not such a potent inhibitor of influenza virus reproduction, is 2,5-dimethyl benzimidazole (35, 36, 37). Like the chloro-substituted riboside, this substance inhibits the intracellular reproduction of influenza viruses but has no effect on extracellular virus particles or their adsorption. In contrast to the dichlororibofuranoside, however, the inhibitory effect is not limited to an early stage in the reproductive process. Although the degree of inhibition is inversely related to the time of addition of the compound, definite inhibition can be obtained during the last part of the latent period (35). This substance also fails to diminish tissue respiration at inhibitory concentration but does restrict cell growth in tissue culture (38). Both the restriction in cell proliferation and the inhibition of virus reproduction are reversible and disappear on removal of the compound. Whether the inhibition of influenza virus reproduction produced by either of these benzimidazole derivatives provides a positive advantage for the functions of the infected cell remains to be demonstrated.

The results obtained thus far with inhibitory derivatives of benzimidazole raise the possibility that a number of processes that lead to the production of new virus particles may be inhibited. The reduction in soluble antigenic material (39), presumably chiefly protein, appears not to be greatly different from the reduction in the yield of virus

particles, which are considerably more complex in constitution and are thought to contain both proteins and nucleic acids. This is dissimilar to the effect of proflavine on the reproduction of phage, which appears to be directed mainly against the final assembly of mature virus particles from a number of preformed components (13). It differs also from the effects of 5-methyl tryptophane or chloramphenicol on phage multiplication, which are considered to result chiefly from inhibition of protein synthesis (5, 10).

If the reproduction of animal viruses is more nearly comparable to that of microbial species than to the stepwise intracellular process now envisioned for phage, the results obtained with the chloro-substituted benzimidazole riboside may not be surprising. Under these circumstances, it might be expected that inhibition of reproduction would be associated with a proportional reduction in all substances found in the virus particle. If, however, reproduction depends on the production of individual components separately and their eventual assembly into mature infective particles, it would seem probable that some component should accumulate when an inhibitory compound is present. There is, so far, no decisive evidence that this occurs with animal viruses.

### *Inhibitors as Chemotherapeutic Agents*

In infections with animal viruses, but not with phage, it is necessary to be concerned with the responses of a society of cells to the results of the reproductive process. Such a community of cells, often of widely dissimilar kinds, constitutes a tissue, and it is the response of tissues that leads to reactions recognizable as disease. In nature, the number of infective virus particles that first succeed in reaching susceptible cells in a given tissue is probably relatively small. The long incubation periods associated with many virus diseases are in accord with this idea. It is well known

that, in experimental animals, the time before gross evidence of infection appears is inversely related to the number of infective particles inoculated. Virus reproduction occurs during the incubation period, and the concentration of new virus particles in an infected tissue increases at a logarithmic rate prior to the appearance of symptoms or signs recognizable as disease (22, 29).

The quantitative relationship between virus concentration and gross lesions, as a function of time after inoculation, is especially well seen in respiratory virus diseases in the mouse. With both influenza virus (22) and pneumonia virus of mice (29), considerable quantities of virus appear in the lung before gross lesions develop. Thereafter, virus concentration continues to increase at a more rapid rate than do lung lesions. Thus, much virus reproduction has occurred before the existence of the infection can be suspected on gross examination. If all the multiplication an infected tissue could support were completed before evidence of disease appeared, it would seem obvious that there could be no hope that substances which inhibit reproduction could be effective as chemotherapeutic agents. Only a fraction of the maximal virus concentration is present, however, when gross lesions appear, and more reproduction occurs as the extent of the lesions increases and the disease progresses (22, 29). There should be, then, an interval after recognition of the disease during which inhibition of virus reproduction could modify the course of the disease.

This hypothesis has been subjected to direct test in infections with pneumonia virus of mice, using *K. pneumoniae* capsular polysaccharide as a chemotherapeutic substance (23). A total of 20  $\mu\text{g}$  of the polysaccharide given either two or three days after inoculation with the virus converts a uniformly fatal disease into one from which the great majority of animals recover. This result is obtained not only when virus reproduction is in the logarithmic



phase and the virus concentration in the lung is considerable, but also when some gross pneumonia has already appeared. On administration of the compound, further multiplication of the agent is inhibited, and the amount of pneumonia does not increase beyond that expected from the preformed concentration of virus. In this particular system, at least, some support apparently can be obtained for the conjecture that a substance which inhibits virus reproduction may exert a therapeutic effect.

Among the difficulties that have appeared, however, are the following: With substances so far employed, the interval between appearance of the disease and administration of the compound cannot be long if a beneficial effect is to be achieved (23). Moreover, the identity of the virus responsible for the disease needs to be known, for inhibitory compounds may show striking selectivity for different viruses in the same host tissue. As an example, *K. pneumoniae* polysaccharides have no inhibitory effect on the reproduction of influenza viruses and do not modify the course of pneumonia induced by these viruses in the mouse (27, 30). This is in sharp contrast to the effects they produce in the same tissue on infections with pneumonia virus of mice (27, 30). It may be that these different viruses do not reproduce in precisely the same cells in the lung and, therefore, that the host cell systems are not identical even though present in a single tissue. But this does not affect the practical aspects of the situation from the viewpoint of the mouse. Under the conditions mentioned, administration of the polysaccharide can modify one virus-induced pneumonia but not the others.

Whether other substances with inhibitory activity will show a similar degree of selectivity with different animal viruses remains to be seen. The number of potentially useful substances so far discovered is discouragingly small. Only a very few such substances have been closely studied with a variety of different viruses, and it is too early to dis-

cern any clear pattern of effects. This is an area of inquiry in which borderline results can be both encouraging and deceptive. Minor extensions in survival time, even though not attributable to chance on statistical grounds, may be so dependent upon the experimental conditions employed as to rob them of any real importance.

Some mention has been made of the short interval that seems to be available for the effective use of inhibitory compounds after evidence of disease has appeared. The idea that this interval is brief has arisen from studies with viruses like influenza, which reproduce at a relatively rapid rate (28). With pneumonia virus of mice, which multiplies less rapidly, an effective interval of at least a day remains after gross signs of disease have appeared (23). Possibly with viruses that reproduce much more slowly, an even longer interval for therapy may be found.

At present, it seems probable that substances which inhibit virus reproduction may come to be more useful during the incubation period than after disease has become manifest. Investigations with a variety of animal viruses, including pneumonia virus of mice (23, 30), mumps (20), and influenza (35, 38), indicate that multiplication is more effectively inhibited the earlier an inhibitory compound is given. As would be expected from a compound that inhibits the reproductive process, the lower the virus concentration when the substance is given, the smaller is the final yield of virus. If the final concentration can be kept below the threshold value, recognizable disease does not appear. Although use of inhibitory compounds during the incubation period is not to be considered as therapeutic, it is also not strictly prophylactic. The virus infection is not wholly prevented but is diminished in extent through reduction in the amount of virus reproduction. In effect, the infectious process is held at the subclinical or inapparent level. The advantage can be double, for not only may evident disease

not result from the infection, but also active immunity against a second infection may develop.

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## Chapter 12

# Challenging Problems in Antibiotic Research

By HARRY EAGLE

The positive accomplishments of antibiotic research, as they relate to perspectives and horizons in microbiology, are difficult to summarize. In part, this reflects the fact that most research in antibiotics has centered around the development of new agents. Beginning with their discovery by large-scale screening programs, and proceeding to their isolation and chemical characterization, the determination of their pharmacologic properties and therapeutic activity, and culminating in efficient methods of production and effective pharmaceutical formulation, the new agents represent an enormous investment of time, facilities, and skills. I do not wish to minimize the importance of such studies, or the value of the chemical and biological data so obtained. Research in that aspect of microbiological antagonism which we term "antibiotic" action was motivated by the development of effective agents for the treatment of disease, and that prac-

tical goal remains its chief inspiration and justification. From the point of view of the microbiologist, however, there is a disheartening disparity between the magnitude of this developmental program in the antibiotics and what is known or even attempted with respect to the elucidation of their mode of action.

### *Practical Applications of Antibiotics*

Let us first look into the present status of the antibiotics at the practical level. Their impact in clinical medicine requires no elaboration. Within a decade, a wide variety of infectious diseases have become amenable to treatment, where previously treatment was ineffective or unsatisfactory. Bacterial endocarditis is no longer a hopeless disease; most rickettsial infections can be rapidly cured with the tetracyclines or with chloramphenicol; the clinical management and prognosis of tuberculosis have been radically altered by the advent of streptomycin; and Ehrlich's dream of a single massive sterilizing dose of a nontoxic compound has been finally achieved in the one-injection treatment of syphilis and other treponematoses with penicillin. Early syphilis is, in fact, rapidly disappearing, to the degree that in many urban areas it is becoming difficult to find case material for student teaching; and yaws, one of the major plagues of mankind, has proved similarly susceptible to treatment with a single injection of penicillin. Parenthetically, I am convinced that the spectacular decline in the attack rate of syphilis is due only in part to the specific treatment of the disease, and in part reflects the haphazard and wholesale use of the antibiotics. Quite unintentionally, this has served to reduce the total reservoir of infection, a desirable by-product of a generally undesirable practice.

Altogether aside from the clinical importance of the antibiotics, their growth-accelerating effects in young ani-

mals and fowl have increased our total meat resources almost overnight, and to a significant degree.

These striking successes and the facility with which the antibiotics can be, and often are, prescribed for the treatment of undiagnosed febrile illness have led to the widespread misapprehension that the problem of infectious disease has been or soon will be solved. The truth falls somewhat short of this optimistic appraisal. The problem of infectious disease has been met only in part, and indeed only in small part. A number of common and serious bacterial infections are not amenable to treatment with any of the antibiotics yet discovered; antibiotic-resistant staphylococci present a special and as yet unsolved problem; the chemotherapy of many protozoal and helminthic infections, world-wide in scope and affecting large segments of the population, is highly unsatisfactory; and although the rickettsial infections and a number of diseases caused by the larger viruses respond satisfactorily to treatment with some of the antibiotics, the smaller viruses are not similarly susceptible.

### *Cell Permeability, a Possible Limiting Factor*

It has been suggested that the small viruses are resistant to therapy because, as intracellular organisms, they cannot be reached by the drugs. But recent studies in our laboratory with radioactive penicillin and streptomycin indicate that at least these two antibiotics do penetrate into some types of mammalian cell in concentrations consistent with a simple diffusion equilibrium. Permeability, as such, may therefore not be the important consideration which limits their therapeutic action within the cell. Conceivably, the larger viruses and the rickettsiae have a more complex structure and more complex metabolic pathways, which offer numerous points of attack for the selective cytotoxic action of, for example, chloramphenicol or a tetracycline,



whereas the smaller viruses may have only a limited metabolic activity and offer correspondingly limited points of attack for a chemotherapeutic agent. If, as appears to be the case with bacteriophage, the metabolic reactions necessary for the replication of the small viruses are in large part mediated by the host cell itself, one must then hope that there are specific metabolic functions which are more essential to the virus than to the host cell, and can be blocked by an appropriate agent. That agent has yet to be found. (Some alternative possibilities and the complexities encountered in the experimental chemotherapy of viral infection are discussed in Chapter 11.)

### *Toxicity of Antibiotics*

Although the toxic reactions noted with every antibiotic are usually minor and transient, in the rare patient they may be serious or even fatal. Despite the increasing number of such case reports, the pathogenesis of the serious hypersensitivity reactions to penicillin, of the gastrointestinal reactions to the tetracyclines, and of the blood dyscrasias sometimes observed with chloramphenicol, remains unexplained and in large measure unexplored; as of now, the occurrence of such reactions can be neither predicted nor prevented.

### *Development of Resistance*

Much attention has focused on the problem of the emergence of resistance to antibiotics. In my estimation, the practical importance of the phenomenon has been greatly exaggerated. True, staphylococci are now more resistant to antibiotic therapy than they were ten years ago. There is reason to believe that this represents not the development of resistance in strains that were originally sensitive, but rather the selective survival and multiplication, under the

influence of antibiotics, of strains that were resistant initially. Except for staphylococci, however, none of the organisms causing infections generally treated with penicillin, chloramphenicol, or the tetracyclines has developed significantly increased resistance; and on the basis of considerations that we will not have time to discuss here, it is unlikely that such resistance will develop sufficiently to interfere seriously with the therapeutic use of these antibiotics. The very real problem of increased resistance to streptomycin during treatment of tuberculosis has been largely met by modified treatment schedules and by the use of para-aminosalicylic acid or isonicotinic acid hydrazide in conjunction with the antibiotic.

Even if the problem of the development of resistance is not so urgent as the lay press would have us believe, it is a problem of broad biologic significance and interest, the mechanism of which has not yet been resolved. There is now an overwhelming body of evidence, stemming from a number of experimental approaches, that the highly streptomycin-resistant organisms which can be demonstrated in any bacterial culture represent spontaneous mutations that occur approximately once in every  $10^8$  cell divisions. It is, however, not equally clear that the slightly increased resistance which develops after exposure to low concentrations of any antibiotic, and which sometimes appears to involve a large proportion of the population, is similarly the result of a spontaneous mutation. The possibility has not been excluded that in the latter case we may be dealing with the adaptation of the organisms to the drug. The difficulty with this explanation is that it implies the inheritance of acquired characters, since this minor increase in resistance may persist for many generations in the absence of the antibiotic. The experimental evidence in favor of adaptation is at best suggestive, but it does warrant further exploration. In the case of penicillin, we have been able to show that the

apparent mass adaptation of large numbers of bacteria to low concentrations in a fluid medium in fact represents the selective multiplication of a few resistant organisms, presumably mutants, introduced with the inoculum. Furthermore, repeated attempts to adapt small numbers of bacteria to penicillin by slowly increasing the concentration of the antibiotic in the medium over a period of days have in our hands been uniformly unsuccessful. On the other hand, with chloramphenicol, there are some experiments which apparently involve no important degree of multiplication or selection and in which an entire bacterial population has apparently become resistant after exposure to threshold concentrations of the antibiotic for 10 to 25 days. Even in these experiments, however, the possibility of a minor degree of multiplication, sufficient to permit spontaneous mutation followed by selective multiplication, has not been rigorously excluded; and there have as yet been no rigorous experiments that prove the adaptation of bacteria to antibiotics.

As to the mechanisms that determine the varying resistance of bacterial species and strains to antibiotics, at least four have now been demonstrated for penicillin alone, and there may well be more. Some bacteria are resistant because they are able to release into the medium an enzyme, penicillinase, which converts penicillin to an inactive form, penicilloic acid. Others that do not release penicillinase into the medium are able rapidly to destroy the antibiotic after it gets into the cell. For most bacteria as they occur in nature, however, the primary determinant of penicillin sensitivity appears to be the affinity of certain penicillin-vulnerable components of the cell and the antibiotic. By using radioactive penicillin it has been possible to show that penicillin-sensitive bacteria have a high combining affinity for the antibiotic, and that relatively penicillin-resistant organisms have a correspondingly lower reactivity. We are not dealing here with a

question of permeability, for cell-free sonic extracts also combine with the antibiotic in relation to the sensitivity of the parent organism. Highly significant in this connection is the fact that no matter what the sensitivity, that is, no matter what the combining affinity with penicillin, bacteria are regularly killed when they have combined with 1.4 to 2.2  $\mu\text{g}$  per gram, equivalent to 1500 to 2800 molecules of penicillin per cell. In four different species, 900 to 1250 molecules were found to be bound per cell without producing a demonstrable effect on growth; when the bound penicillin reached 1500 to 1700 molecules per cell, there was a marked inhibition of growth; and the bactericidal action reached maximal levels when the bound penicillin was 1500 to 4000 molecules per cell. Bacteria as they occur in nature, therefore, only appear to differ in their sensitivity to penicillin. They actually differ with respect to the concentrations that must be present in the outside medium to effect the uniformly lethal degree of combination with the cell.

Here, then, is the mechanism of selective toxicity originally postulated by Ehrlich, a selective toxicity not based on differences in growth requirements, metabolic pathways, or cell permeability, but rather based on differences in the chemical reactivity of a vulnerable cell component in the resistant and sensitive species.

The nature of the penicillin-reactive component and of the metabolic reaction that is thereby inhibited remains to be determined.

The relative nontoxicity of penicillin for the mammalian host apparently rests on a similarly weak combining reactivity between penicillin and the cell. As in highly resistant bacteria, the concentrations inside the cell are regularly less than those in the surrounding medium and are consistent with a simple diffusion equilibrium. Most of this cellular "penicillin" is readily eluted on washing, is actively bactericidal, and is presumably the free and

unchanged antibiotic. Finally, in contrast to penicillin-sensitive bacteria, and like highly resistant organisms, cell-free extracts of mammalian cells bind the antibiotic to only a limited degree.

The foregoing discussion relates to bacteria as they occur in nature. Other mechanisms of resistance come into play with resistant variants of naturally sensitive bacteria. Such resistant variants usually bind penicillin to at least the same degree as does the sensitive parent cell, but an ordinarily lethal degree of combination now has no effect on growth and multiplication. Their resistance may therefore involve one or more mechanisms qualitatively distinct from any of those previously discussed. The importance of similar studies with radioactive streptomycin, chlortetracycline, and chloramphenicol requires no elaboration.

### *Mode of Action*

Perhaps the widest gap in our understanding of the antibiotics relates to their mode of action. For each of the important antibiotics, a number of suggestions have been made, each based on experimental observations. This very multiplicity of theories suggests that none may represent the primary locus of action of the antibiotic. The striking accumulation of uridine phosphates in penicillin-treated bacteria; the puzzling observation that the binding of penicillin does not initiate the death of the organisms unless that binding occurs in a medium favorable for growth; the inhibition of adaptive enzyme formation by a number of antibiotics; the elimination of chloroplasts from *Euglena* by streptomycin, and of the Kappa-factor from paramecia by chloramphenicol; the uncoupling of oxidative phosphorylation in mammalian tissues by chlortetracycline—these are only a few of the many significant clues from a number of laboratories. But as yet they have not led to a definite explanation for the mode of action of any one

antibiotic. The sometimes striking antagonistic and synergistic effects when antibiotics are used in combination are of obvious central importance in relation to their therapeutic use, but the factors underlying those effects are almost wholly obscure.

Many of the reported metabolic effects of antibiotics are observed with whole-cell suspensions, in which it is notoriously difficult to distinguish between a primary locus of action of the antibiotic and metabolic effects resulting from that combination which may, however, be two, three, or more steps removed. Uncommon interest therefore attaches to the recent demonstration by Saz that in a cell-free bacterial extract, chlortetracycline in minute concentration inhibits an aryl nitro-reductase of *Escherichia coli*. This inhibition apparently results from the fact that the antibiotic combines with Mn, which is an essential cofactor in one or more of the multiple enzyme systems involved in this complex reaction. Interesting also is the fact that oxytetracycline, although it also chelates with Mn, is far less inhibitory than is chlortetracycline. Whether or not this is the mechanism by which chlortetracycline exerts its growth-inhibitory effect on bacteria, it is the first clear demonstration of a direct effect of an antibiotic on an enzyme system in a cell-free bacterial extract and, as such, may prove a model for the actual mode of action of this and other antibiotics.

Our ignorance extends not only to the mode of action of antibiotics at the cellular level, but indeed to how they work *in vivo*, for some of the antibiotics, notably chloramphenicol and the three tetracyclines, are effective therapeutically at concentrations in the body fluids which are only growth inhibitory *in vitro*. The therapeutic response clearly involves the participation of host mechanisms to an important degree; but whether we are here dealing with a humoral or a cellular effect has not yet been clarified.

### ***Physiological Significance to Producing Organisms***

Waksman and his associates have stressed yet another important and neglected area of antibiotic research. What is the physiological significance of the antibiotics to the organisms that produce them. We assume there is but little reason to believe that the antibacterial action of antibiotics gives a selective advantage to the producing organisms in the particular environment in which these organisms occur in nature. There is a reasonable possibility that if some of the time and effort now spent in the screening of tens of thousands of mold filtrates for antibiotic activity were diverted into a study of the metabolic reactions by which these biologically and chemically unique compounds are produced, information of practical as well as academic interest might accrue.

### ***Chemotherapeutic and Microbiological Horizons***

I am afraid this presentation has emphasized some negative aspects of antibiotic research, rather than its striking positive accomplishments. For the physician and for the drug house, the horizon with respect to antibiotics is described in terms of new agents, with an even broader spectrum of activity and an even lower order of toxicity; in other words, in terms of prospective cures for diseases not now susceptible to treatment. Large resources of personnel, facilities, and funds are now dedicated to that end, and usually involve the mass screening of mold filtrates. Over the last ten years, that empiric approach has been enormously rewarding. But what to do when the rich treasure lode of the *Streptomyces* has been exhausted? At least with respect to antibacterial agents, that prospect is already upon us. When a new mold is now found to produce a nontoxic antibiotic, that antibiotic almost invariably

proves to be streptomycin, chloramphenicol, or a tetracycline. A fresh approach and a new orientation may prove necessary if these screening programs are to be of continuing productivity. Whether that fruitful new approach involves an intensive study of the metabolic pathways by which the antibiotics are produced; whether the screening program itself could be profitably modified, for example, by greater emphasis on protozoa and viruses; or whether the chemical modification of known antibiotics, with a view to altering their antibacterial spectrum or reducing their toxicity, deserves further exploration; this is the multiple-choice problem with which the pharmaceutical industry has been faced for some years.

We as microbiologists cannot share the satisfaction of the physician and of the drug house in their joint past accomplishments. However reluctantly, we must admit that the striking chemotherapeutic advances of the past decade have resulted from screening programs. There has been no comparable progress in the development of new agents on the basis of *a priori* considerations, or even in our understanding of the cellular effects and selective cytotoxicity of the known agents. This is the intellectual challenge of the antibiotics. Even on a pragmatic level, it is possible that in this instance also, to paraphrase the title of Flexner's famous essay, the slow and laborious pursuit of apparently useless knowledge for knowledge's own sake may in the long run prove even more rewarding than the screening programs have proved to date.



## Chapter 13

# *Microorganisms and Plant Life*<sup>1</sup>

By ROBERT L. STARKEY

Broadly viewed, the relationships between microorganisms and plant life include much of the area of soil microbiology, for nearly all activities of microorganisms in soil affect plant development directly or indirectly.

Personal considerations may dictate our greater concern with the relations of microorganisms to animal life than to higher plants, for our environment supports a host of microorganisms inhospitable to humankind. Nevertheless, a strong case can be made for the view that plants are so intimately associated with the welfare of the animal that they are deserving of at least equal consideration. Lest we become too absorbed in our own importance, let it be recalled that among living things man is dependent

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on plants, whereas plants can develop in the complete absence of man and even other animals. As regards dependence, Dodge (9) made the following case for fungi: "We may rest assured that as green plants and animals disappear one by one from the face of the globe, some of the fungi will always be present to dispose of the last remains." To continue a bit further, both fungi and most bacteria would encounter nutritional difficulties with the loss of green plants and animals. In this connection, a good case could be made for chemoautotrophic bacteria as the organisms most likely to survive the longest in a world being depleted of living things.

My discussion is based on the principle that higher plants are indispensable to human welfare and that microbial development is intimately related to plant growth. Whereas most microorganisms grow separately from plants, others are associated in states of symbiosis and parasitism.

### *The Rhizosphere*

Among the relations of microorganisms and plants, most attention has been devoted to the role of microorganisms as converters of soil materials, frequently summarized in cycles of the elements. In addition, attention has been directed to structural modification of soil by microorganisms. These changes do not require close association between microorganisms and higher plants, yet the association is frequently closer than may be realized, and the effects of the microorganisms on the plants are pronounced, for the organisms are in close proximity.

The area of the soil close to plant roots was designated many years ago by Hiltner as the "rhizosphere." This is the region of the soil from which the plant obtains its nutrients and in which each soil exerts its particular effects on the plant. In this zone, one finds great numbers of microorganisms, far greater than in the rest of the soil (39).

Bacteria may be 5, 10, 20, or more times as abundant close to or on root surfaces as in soil close by but free from root development. The effects are evident on root hairs and young roots as well as on older roots and are most prominent during periods of active plant development. Furthermore, upon death of the plant, the bacterial population reverts promptly to its original low level. Studies of the rhizosphere were initiated in our laboratories some years ago and have been expanded by many others, particularly by Lochhead and associates (19) and by Clark (5).

The rhizosphere effect has two general implications: first, the extensive development of microorganisms in the rhizosphere is the result of plant growth and is due principally to organic materials coming from the roots; second, microorganisms that have made extensive development on the root surfaces have important effects on the plants because of products formed close to the absorbing root surfaces.

Unfortunately, there is insufficient information to show precisely what happens at the root surface. It is presumed that considerable amounts of readily decomposable organic materials come from the intact roots, and they, together with diverse dead root parts, support this large population of microorganisms. There is only fragmentary information, however, as to whether organic materials are excreted from roots, and little to indicate what the compounds are and the conditions under which they are excreted. There is some evidence that root penetration by the nodule bacteria is conditioned by root excretions, and Virtanen and his associates made proposals regarding the mechanism of nitrogen fixation by leguminous plants on the basis of the amino acids excreted from root nodules (42, 45). Some information presented by Halleck and Cochrane (14) was believed to indicate that certain fungistatic substances applied to the aerial parts of plants affected the development of bacteria in the rhizosphere, presumably through

excretion of either the toxic materials that had been translocated, or through other organic materials excreted in response to effects of the fungicides on plant development. Some of the substances decreased the numbers of bacteria in the rhizosphere, whereas others increased them.

More definite evidence of movement of organic materials through plants was recently reported by Preston, Mitchell, and Reeve (32). When the tops of certain plants were treated with  $\alpha$ -methoxyphenylacetic acid and the treated plants were grown in the same pot with untreated plants, the chemical moved downward through the treated plants, out of the roots, and was taken up by the others. When grown in solutions, the treated plants liberated the chemical into the water. The effect was noted only with this one plant-growth-modifying compound and not with others, yet it establishes the possibility of the excretion through roots of organic materials applied to aerial plant parts. Winter (47) has included the following among the organic materials excreted by plant roots: phosphatides, amino acids, thiamin, biotin, mesoinositol, para-aminobenzoic acid, carbohydrates, tannins, and alkaloids. Harley (15) listed sugars, amino acids, vitamins, and other organic substances as materials excreted by roots. The total number of excreted substances must be large. Obviously, more information on the movement of organic materials from plant roots is needed.

Under conditions of high air humidity and low content of soil moisture, the plant may affect microbial development in still another way. Breazeale and McGeorge (1) reported that, under these conditions, water is transported from the air through the plant to the rhizosphere, and in this way the soil moisture content can be increased. In one of their experiments, tomato plants grown in soil in which the water content had been reduced to the point where the plants began to wilt were placed in an atmosphere of 80 to 90 per cent humidity. The plants recovered

and continued to grow and bear fruit with no watering except that provided by the fog.

Somewhat more is known of the effects on plants of microorganisms in the rhizosphere, but the data are little more than suggestive. The solvent effect of roots on marble was shown by plant physiologists many years ago, and it was found by Fred and Haas (10) that the solvent effect was greater when microorganisms were present than when they were absent. The effect was ascribed to carbon dioxide excreted from the roots. Of particular significance in the same connection are some results of Gerretsen (11) on the solubility of phosphate. It was found that more tricalcium phosphate was absorbed by plants from substrates containing microorganisms than from sterile substrates, and that the phosphate underwent solution by microbial development about plant roots.

Solubility of iron, and also of manganese (12), and their availability to plants are probably affected by the rhizosphere population. Microbial development reduces the redox potential and may increase the acidity, both of which increase iron solubility (40). Recent results of Bromfield (3) support this contention.

Although reduction reactions play an important part in the solution of iron and manganese, complex formation may affect the availability of these elements to plants as well. Among the diverse organic compounds formed during development of microorganisms, presumably there will be chelating materials which will persist long enough to form complexes with iron and other metals, and these complexes will enter the plant. Amino acids, citric acid, and other microbial products could serve as chelating agents. Penetration of chelating compounds should be no problem and would be likely to aid in iron utilization. Recent observations of Weinstein *et al.* (44) have shown that, when part of a root system was placed in a solution containing the chelating agent ethylenediaminetetraacetic

acid (EDTA), and part in a solution containing iron, the iron was mobilized, whereas in the absence of EDTA it remained largely immobilized in the roots as ferric iron. The production of chelating agents by microorganisms in soil and the significance of these compounds in the metal nutrition of plants are still in the area of speculation.

Although plants can absorb certain organic compounds, the extent of this absorption under natural conditions is uncertain. But soil organic matter may be a source of organic as well as mineral substances for plants. Miller (24) found that tomato plants could satisfy their entire sulfur requirements from the amino acid methionine. Various antibiotics are absorbed by higher plants, as are the auxin indolylacetic acid, various plant growth regulators and herbicides, fungicides, and insecticides. The uptake of certain of these compounds is cause for concern because such compounds may affect the quality of the plant and its acceptability as food for man and domestic animals.

The possibility that many organic substances of microbial origin in soil, particularly in the rhizosphere, are absorbed and affect the plant, or its food value, favorably or unfavorably is still to be evaluated.

There is still another manifestation of the effects of organic materials of microbial origin on plant development. Cholodny (4) reported that plant roots, when exposed to soil gases, showed abnormalities indicative of effects produced by some volatile organic substance of microbial origin. The possibility that this was due to gaseous hydrocarbons gains support from recent experiments of Davis and Squires (8). They grew mixed cultures of microorganisms from cow manure and from sewage sludge, as well as a culture of the fungus *Penicillium digitatum*, in substrates containing carbohydrates or salts of organic acids. In addition to methane and ethylene, the products contained the hydrocarbons ethane, propane, and propylene, not previously reported as being formed by micro-

organisms. Some of these can be expected to affect root development in appropriate concentrations.

In some respects the root surface of the plant, the "rhizoplane" of Clark (5), resembles the digestive tract of animals. Both are the principal regions of absorption of various substances from the external substrate; in both cases there is extensive development of saprophytic bacteria, and in both cases organic materials affecting the microorganisms are liberated from the absorbing system into the region of microbial development. The microorganisms act on the substances in the external substrate, decompose some of the compounds, produce others, and affect the nutrient value of the substrate.

### *Development of Microorganisms within Plant Tissues*

Among the more intimate associations of microorganisms and higher plants are symbiotic phenomena designated as "bacteriorrhizae," when bacteria are involved, and "mycorrhizae," when filamentous fungi are concerned. In both cases the microbial associate penetrates the plant tissues, where it is supported in part or entirely by the plant, and the plant generally benefits from the associative development of the microorganism.

#### BACTERIORRHIZAE

The association between legumes and bacteria of the genus *Rhizobium* is the best known and virtually the only well defined example of bacteriorrhizae. The bacteria become located within the plant in the tissues of the specialized root nodules, and they depend entirely on the conditions of environment and food supply provided by the plant. Once they have entered the plant, they are affected by the soil conditions only indirectly, through the influences of these conditions on development of the plant.

One can indicate what seems to be a logical sequence of events from the time the bacterium affects the root hair and gains entrance, until the nodule is developed and the relationship between the bacterium and the plant is consummated (45). The story is far from complete, however. One may inquire: why do members of the genus *Rhizobium* develop an association with legumes, whereas other bacteria are unable to do this? Furthermore, what is it that prevents not only legume bacteria, but all bacteria from developing symbiotically with other plants? These questions have puzzled microbiologists and plant physiologists since the time that nitrogen fixation was found to be the result of the symbiosis. We seem little nearer to the answers now than before.

With a few exceptions, such as certain leaf endophytes and the association between bacteria and legumes, the tissues of higher plants appear to be unfavorable for bacterial growth. This is particularly the case with the aerial portions of the plants. An interesting contribution to the subject was made by Russell (37) before the turn of the century. In his dissertation at Johns Hopkins University in 1892, he reported on the effects of injecting cultures of bacteria into stems of plants. Although some of the saprophytic bacteria persisted for more than a month and seemed to multiply, they failed to destroy the tissue. Animal pathogens survived in tissues for much shorter periods. Most of the penetration of the bacteria was upward. This varied from 30 to 50 mm. Downward movement never exceeded 2 to 3 mm from the point of inoculation. The following citation indicates that he found the plant an unfavorable substrate for saprophytic bacteria: "From the results of my own experiments, the conclusion seems evident, that, normally, the healthy plant, with intact outer membranes, is free from bacteria within its tissues."

Bacteria have been recovered from plant tissues by various investigators since the studies made by Russell. They



are more readily recovered from roots than from stems and leaves. Little significance can be attached to their presence, however, for they occur in small numbers, and their presence seems to be unrelated to plant vigor or survival.

#### MYCORRHIZAE

Mycotrophy in plants can be interpreted broadly as the associative development of fungi and plants, in which the fungi penetrate the plant tissue (16, 20, 22, 33). It is a very general phenomenon in nature and has been reported for all of the major groups of plants, including the algae, mosses and liverworts, ferns, gymnosperms and angiosperms. Particular attention has been devoted to the associations of fungi with orchids, forest trees, and ericaceous plants. There are two major types of fungus development: the ectotrophic, in which the fungus makes profuse growth about the root surface and develops between the cells of the epidermis; and the endotrophic, where there is little external mycelium but the fungus penetrates deeply into the tissues and invades the cells. There is also a type referred to as ectendotrophic, where the fungus development is intermediate between the two. The significance of development of the fungus associates has been variously interpreted, and it is probable that with different plants the effects of the fungi differ. In plants that have limited, if any, root systems, the fungus functions as root hairs. Kelley (20) has said: "It is unknown whether plants in nature have root-hairs or mycorrhizae, or neither; but there is enough evidence at hand to indicate that mycorrhizae predominate over root-hairs in the majority of cases." In chlorophyll-free plants, the fungi may provide the plant with organic materials. With pines and certain other trees, the fungi cause the development of an abundance of short roots and thus provide greater absorbing surface (15, 16, 17). Furthermore, minerals become absorbed through the fungus mycelium which serves as root hairs (23).

In some cases benefits have been reported from inoculation of young trees with the fungus endophytes, and poor development of trees in certain areas has been ascribed to lack of the necessary fungi. One of these cases was reported by Rayner and Neilson-Jones (35) for an area known as the Wareham Heath in England. Inoculation with mycorrhiza soil, or addition of compost, or sterilization and subsequent inoculation corrected the deficiency. It was concluded that some biological factor limited development of the fungi. The possibility that this factor was an antibiotic active against the mycorrhizal fungi has been proposed by Jefferys (18), Wright (48), and others (2, 34).

There is agreement that mycotrophy is important, that it is of common occurrence, that it has significance to the plant host, and that the fungus makes limited development in the plant, controlled in some way by it. This is almost the limit of agreement. Whereas there is evidence of some degree of specificity between the fungus species and the plant with which it is associated, this may not be a fixed relationship with all mycorrhizae, for it has been said (20), "There are no mycorrhizal fungi, there is only a mycorrhizal state."

In one of the recent reviews of mycotrophy, Kelley concluded that the endotrophic type is most common. In this type of association the fungus penetrates the cells where the filaments undergo intracellular digestion, releasing their contents. The fungus derives some materials from the plant, and the plant obtains some substances from the fungus, but the nature of the substances and their importance to the organism that receives them are obscure.

The diversity of types of mycorrhizae, as well as the variety of the plants and fungi involved, has contributed to the confusion regarding the importance of the fungi to the hosts.

### ***Parasitism***

Mycotrophy has been interpreted by some investigators as parasitism, but, if so, development of the parasite is limited and is controlled by the host. Extreme parasitism, resulting in diseases of plants, is a characteristic of many microorganisms, including various bacteria and filamentous fungi. Although the number of pathogens is large, it is not evident why the number is not greater and why there is such specificity between parasite and host. Although the surface structures and other anatomical characteristics of plants may provide some protection, it is unreasonable to believe that these barriers are the principal means whereby plants are protected from destruction by microorganisms. The fact that the resistance of some varieties of plants is correlated with anatomical differences, such as epidermis composition or thickness, has limited significance in explaining the general basis of plant resistance (46). In this connection, Orton (27) said: "It is hard to understand why a thick cell wall should protect from infection a leaf which has many thousand openings as breathing pores through which a fungus might enter . . ." Furthermore, mention has already been made of both intracellular and intercellular penetration of plant tissues by microorganisms where the degree of penetration has been controlled apparently by something other than physical structures.

It is particularly difficult to understand how plant roots remain vigorous when they are coated with cells of microorganisms, including types able to decompose the various substances of which the roots are composed. These root structures should be ineffective barriers against penetration by such soil microorganisms. Certain organic substances such as tannin, catechol, protocatechuic acid, other phenolic substances, alkaloids, organic acids, and esters may serve to protect specific tissues, but it has not been shown

that their distribution in plant tissues is sufficiently general to provide immunity from destruction (46).

The fundamental basis of resistance of plants to microbial attack is still to be disclosed. In the words of Coons (6): "We do not know at all what makes one plant less susceptible than another, nor do we know the basis of fungus specificity that makes one species, genus, or family of plants completely immune from a given parasite." This might be generalized to include all kinds of microorganisms as well as higher plants. The ability of plants to survive in contact with microorganisms, and of microorganisms to develop in contact with one another, must depend on compounds that prevent parasitic attack. Sherman and Hodge (38) obtained a clue to the existence of such compounds from the antibacterial action of expressed juices of cabbage and turnip. Antimicrobial substances, designated "phytoncides," have been detected in many plants. The fact that these substances do not seem to be present in all plants, and that antibiotics are not presumed to provide protection to the cells that produce them, suggests that the substances that provide resistance are unknown. It remains to be established whether they are alike or dissimilar in various plants and microorganisms, and whether they are relatively stable compounds or must be continuously regenerated or restored to an active state. The fact that killed plants are very susceptible to microbial attack tends to support the latter possibility.

### *Antibiotics and Plant Development*

Before concluding my remarks, I should like to mention briefly the relation of antibiotics and plant development: that is, the production of antibiotics in soil and their effects on the soil population, their uptake by plants, their influences on plant development, and their use to control plant diseases.

Even though many different microorganisms able to produce antibiotics on laboratory media can be obtained from soil, this provides no indication that these same organisms will produce antibiotics in soil. In fact, it is difficult to demonstrate antibiotic production in soil (41). In several cases, demonstrable amounts of antibiotics have been produced in sterilized soil that was treated with organic substances and inoculated with pure cultures of the antibiotic-producing microorganisms. Antibiotic production in unsterilized soil has been demonstrated infrequently. Gregory, Allen, Riker, and Peterson (13) achieved production of antibiotics by various microorganisms in unsterilized soil supplemented with alfalfa meal or straw. Wright (48) obtained gliotoxin under similar conditions when clover was added to the acidic podzol of the Wareham Heath. These difficulties in producing antibiotics, even under conditions which should be more favorable than those common to soils in nature, suggest that antibiotics are unlikely to be present in soils in appreciable amounts except in micro areas.

Under conditions where the amounts of antibiotics are low and localized, the likelihood that antibiotics will exclude certain elements of the soil population is small. The relationships of bacteriophage in soil may be comparable. If a phage contained in a soil controlled the population, one might expect to recover only phage-resistant cells, if any. But phage-sensitive cultures are obtainable from soils that contain the phage. Probably, biological control by such factors as phage or antibiotics would be localized, temporary, and limited in effect except under unusual conditions.

Furthermore, results obtained in our laboratories (31, 41) indicate that the amounts of antibiotics required to affect microorganisms are much greater in soil than in culture media, and that even streptomycin, which is very resistant to decomposition in comparison with other anti-

biotics, undergoes fairly rapid destruction in soil (30). Although this evidence does not exclude the possibility of some control of the soil population by antibiotics, particularly root disease microorganisms, it limits very much the conditions under which the population can be controlled.

Provided that antibiotics are available, they can be absorbed and translocated through the plants. Here is one more evidence of absorption of organic materials by plants. Pramer (28, 29) and Crowdy and Pramer (7) observed that basic materials move less rapidly than neutral or acidic ones and that uptake is more rapid by cuttings than by rooted plants. Furthermore, absorption and translocation vary with the plant species and the environmental conditions. Once absorbed, streptomycin and griseofulvin, and doubtless others, persist in plants. Active material, which was presumably the streptomycin with which the plants were treated, has been detected in plant tissue ten weeks or more after treatment (36).

Nickell (25, 26) reported that the addition of certain antibiotics to soil resulted in increased growth of plants during early stages of development and that antibiotics increased germination of some seeds.

This type of response awaits confirmation,<sup>2</sup> but there is ample evidence that many plant diseases can be controlled by use of antibiotics (21, 43). In our laboratories, Robison has successfully controlled a bacterial wilt of chrysanthemum with streptomycin (36). Many of the antibiotics are effective systemically. Treatment for control of plant diseases may consist of adding the antibiotic to the seed or to the cut shoot, to the soil or sand substrate, or as a spray or dust to the aerial parts of the plants. Downward movement through the plants is slow, and this limits treatment of the top parts of plants for control of root infections. The potentialities of antibiotics for control of plant diseases appear

<sup>2</sup> In a recent report Barton and MacNab (Effect of antibiotics on plant growth. *Contrib. Boyce Thompson Inst.*, 17:419-434, 1954) observed little or no favorable effect of antibiotics on germination of seeds, growth of seedlings, or development of plants in soils.

promising but are as yet little known. The attention given to the effects of antibiotics on plants and plant diseases is slight compared to that given to their effects on animals and animal diseases.

### Concluding Remarks

In this paper, some of the varied relationships between microorganisms and higher plants have been indicated, and some of the limits of information regarding these relationships have been pointed out. From even a cursory consideration, it is evident that the relationships are numerous and diverse. A comprehensive discussion would include many types of associations not mentioned at all here. Although something is known of many types of associations, knowledge of many, and indeed of most of them, is limited. The lack of information concerning the reactions involved and the factors affecting the associations might well serve as a challenge for present and future investigators.

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APPENDIX

ADDRESSES DELIVERED AT  
THE DEDICATION OF  
THE INSTITUTE OF  
MICROBIOLOGY,  
RUTGERS UNIVERSITY,  
June 7, 1954



# *Dedication of the Institute of Microbiology*

By LEWIS WEBSTER JONES  
President, Rutgers University

We are met here to dedicate a building, this magnificent Institute of Microbiology, and to rededicate this university to the high purposes which this building symbolizes.

For Rutgers University, this is a proud and solemn moment. We recognize gratefully the work and achievements of one of the great creative personalities of our age and Rutgers' most distinguished son, Dr. Selman A. Waksman. He was educated at Rutgers, and most of his scientific work has been carried on here. His great abilities, his long and patient investigations, his skilled and inspiring leadership have brought honor to his university and untold benefits to humanity. He has used the material rewards of his discoveries, with characteristic unselfishness, to further the scientific work to which he has devoted his life.

This university gains immeasurably from the presence

of this great international center of investigation and teaching, and accepts with enthusiasm, gratitude, and resolution all the responsibilities it implies.

We welcome the scientists from many foreign countries who are with us today. Their presence emphasizes the international tradition of science, world-wide in its scope and in the promise it holds for the destiny of the human race. The language of science is truly international, truly humane in its purposes. Science seeks to arrive at verifiable knowledge. In striking contrast to the struggle of rival propagandas that poison communication in the political sphere and erect impenetrable barriers between peoples, communication among scientists means a sharing of truth; all the evidence is carefully checked and openly presented for the common enlightenment. Scientific investigation is an expression of a continuing faith in human reason, in man's power to increase his dignity and stature by increasing his understanding.

In the dreary climate of the "cold" war, our vision of the future has been clouded, and its range sadly shortened by the fogs of doubt, fear, and suspicion. We can be thankful, as we contemplate this Institute of Microbiology, for the clear, bright prospect it opens before us. We can be sure that the work that will be done here will be more significant, more far-reaching, and infinitely more creative and enduring than the political events that fill today's newspaper headlines.

This institute will be hospitable to scientific workers and students from many lands and from many related fields of investigation. We are dealing here with the most constructive powers of the human mind; and the creative imagination of the scientist links him in a common fellowship with artists, poets, philosophers, and all men of learning and good will, with all indeed who share the aspirations of the spirit.

This will be a place where the imagination of gifted

individuals will have full scope; where teams of scientists can pool their resources for the advancement of knowledge. It will give mature workers an opportunity for continuing achievement, younger workers a chance to learn and develop their skills. It will be mainly concerned with basic investigations into the nature of microorganisms, as well as with the many and varied applications of newly discovered knowledge. It will be truly productive, in a world too much threatened with destructive forces; productive of understanding, enlightenment, and practical human welfare.

We therefore dedicate this building, and rededicate this university, to the enduring values on which our civilization rests: the search for knowledge and understanding, and their humane use under the guidance of universally applicable ethical principles. We thus reaffirm our faith in reason and good will and our hope for the future.

# *Microbiology Takes the Stage*

By SELMAN A. WAKSMAN

Director, Institute of Microbiology

The first institute devoted primarily to microbiology was named after Louis Pasteur. It was dedicated two thirds of a century ago, November 14, 1888. At the time of its organization, the staff consisted of Pasteur as the director; Duclaux, in charge of general microbiology; Chamberland, concerned with microbiology in relation to hygiene; Roux, studying microbiological methods in their medical applications; and Metchnikoff, working in the general field of morphology of lower organisms and comparative microbiology.

These investigators were interested primarily in the relation of microorganisms to human health and the causation of disease. General microbiology, systematics of microorganisms, and microbial physiology and biochemistry occupied only a secondary place. Microbial genetics and cytology were hardly considered. The broad aspects of the role of microorganisms in the cycle of life in nature and in soil processes and the wider problems of chemotherapy were not yet contemplated.



We have come a long way during these last few decades. There is no field of human endeavor where microbes are not recognized now as playing an important, and frequently a dominant, part. We can now recognize, utilize, combat, and in most cases control the numerous microbes that cause infectious diseases and epidemics, that attack our animals and our plants, that destroy our food supplies and our clothing, that inhabit our soils and our seas, the air we breathe, the water we drink, and even our bodies. We also have learned to know microbes as complex living systems. We now understand better their growth and their functions, their manner of multiplying, and of utilizing simple and complex nutrients, and we have gained considerable information concerning the complex biochemical reactions involved in these processes.

We have learned much about the life of microbes in pure cultures and in complex populations, and about the production by many of them of specific chemical substances which stimulate or inhibit the growth of others. We have learned also to utilize such substances for improving and accelerating the growth of higher forms of life, for destroying injurious microbes, thus improving human and animal health. Diseases and epidemics that formerly decimated huge segments of the world's population have now been brought under control, deaths due to diseases of childhood are now relatively rare, and the life span of man has been greatly extended.

How has all this been brought about? What have we learned that will help us to plan our future investigations? To what extent has the microbiologist benefited from the contributions of the general biologist? In what respects do the methods of studying bacteria, fungi, actinomycetes, and protozoa differ from the methods of studying higher forms of life, both plant and animal? Are the tools, the methods, the approaches, or observations used in the study of microbes different from those employed by the general biologist?

These and many other questions that pertain to the world of microbes in which we live have been only partly answered. Many questions still await the investigator. These problems are fundamental in nature, but the answers promise rich rewards in practical applications. Many questions have remained unanswered because the proper methods of investigation have not yet been discovered. We are dealing with microscopic and ultramicroscopic forms of life, which we often recognize not by what they look like but by what they do; not by how they behave in a normal environment, but by how their reactions in an abnormal environment can be interpreted in terms of their life processes and role in the cycle of life in nature.

Claude Bernard emphasized the fact that reasoning is correct only when applied to correct facts; when facts are originally tainted with error and inaccuracy, reasoning based on them can lead only to error: "The art of investigation is the cornerstone of all the experimental sciences. If the facts used as a basis for reasoning are ill-established or erroneous, everything will crumble or be falsified; and it is thus that errors in scientific theories must often originate in errors of fact." If this applies to higher forms of life, it applies with even greater force to the life of the microbes. Bernard stated further: "The investigator must be at once a theorist and a practitioner. He must completely master the art of establishing experimental facts, which are the materials of science, and he must also clearly understand the scientific principles which guide his reasoning through the varied experimental studies of natural phenomena. Head and hand must go together. An able hand without a head to direct it is a worthless tool; the head is powerless without its executive hand."

We can subscribe heartily to these principles. Pasteur himself said at the dedication ceremonies of his institute: "My dear collaborators, keep your enthusiasm, but let its inseparable companion be rigorous control. Do not ad-

vance any idea which cannot be proven in a simple and decisive manner. Cultivate the critical spirit. In itself, it is neither a provoker of ideas nor a stimulant to great things. It always has the last word, however."

There has been much elaboration on Pasteur's dictum, "Chance comes only to the prepared mind," or as Nicolle expressed it, "Chance favors only those who know how to court her." Helmholtz said, "Happy ideas come unexpectedly without effort, like an inspiration. So far as I was concerned, they have never come to me when my mind was fatigued, or when I was at my working table. . . . They came particularly readily during the slow ascent of wooded hills on a sunny day." The poet Byron expressed it in somewhat different terms: "To be perfectly original one should think much and read little, and this is impossible, for one must have read before one has learnt to think." Theobald Smith emphasized that "Discovery should come as an adventure rather than as the result of a logical process of thought."

The ability of the investigator to discriminate is another aspect of the methods of discovery that has often been neglected. It may not be so spectacular as chance but is more certain to bear fruit. This was well expressed by Hans Zinsser: "The scientist takes off from the manifold observations of predecessors, and shows his intelligence, if any, by his ability to discriminate between the important and the negligible, by selecting here and there the significant stepping-stones that will lead across the difficulties to new understanding. The one who places the last stone and steps across to the *terra firma* of accomplished discovery gets all the credit. Only the initiated know and honor those whose patient integrity and devotion to exact observation have made the last step possible." Paul Ehrlich emphasized the need for "much testing, accuracy and precision in experiment, and no guess work or self-deception."

To envisage the progress of science, we may again refer

to Bernard, who pointed out: "New ideas and discoveries are like grains: it is not sufficient to produce them and seed them; they must be nourished and allowed to develop by means of scientific culture. Otherwise, they die or emigrate to other places where they are found to prosper and bear fruit wherever they find fertile soil, often far away from the place of origin." Also, he emphasized that "men who have excessive faith in their theories or ideas are not only ill-prepared for making discoveries, but they also make poor observations."

The scientific method is clearly outlined by Cannon in *The Way of an Investigator*. He says, "Curiosity has been condemned as a disease and as a low vice, and the theologians and poets have solemnly warned against it. But in spite of the testimony that it was curiosity which lost us paradise, I am sure that all who are aware of the fruits of the tree of knowledge would agree that they have become abundant because of the spying and trying of inquisitive scientists. . . . [The investigator] sees events and changes in his field which seem to him strange and mysterious. Instead of ignoring them, as most people do, he wonders about them and sets to work to learn their characteristics. Curiosity is the main-spring of his initiative and his persistent industry. It is a prime requisite for a career of exploration."

The investigators themselves deserve particular attention. In his introduction to Max Planck's *Where is Science Going*, Einstein emphasizes that there are ". . . many kinds of men who devote themselves to science, and not all for the sake of science itself. There are some who come into her temple because it offers them the opportunity to display their particular talents. To this class of men science is a kind of sport in the practice of which they exult, just as an athlete exults in the exercise of his muscular prowess. There is another class of men who come into the temple to make an offering of their brain pulp in the hope of se-

curing a profitable return. These men are scientists only by the chance of some circumstance which offered itself when making a choice of career. . . . Should an angel of God descend and drive from the Temple of Science all those who belong to the categories I have mentioned, I fear the temple would be nearly emptied. But a few worshipers would still remain. . . . For the most part they are strange, taciturn and lonely fellows. And, in spite of this mutual resemblance, they are far less like one another than those whom our hypothetical angel has expelled."

This brings me to the methods of educating scientists. I am not primarily concerned with secondary or even undergraduate college education. I have in mind graduate training, the education of the future scientist after he has definitely decided upon a field of work and often even upon a type of problem, or at least upon a method of approach to a problem.

Some educators fill the candidate with information. In time, they make him feel that he knows all there is to be known about a certain field of science, and sometimes all that will ever be known about a given problem, or the approach to the problem. This is, of course, the way to the "expert," the object of hero worship, and often even to that of the mystic in science. Other educators make the candidate feel that he knows only very little, in fact, that no one knows very much in a given field of science, and that the future lies before him to be investigated.

The first method is by far the more satisfying to the student. He completes his work with a feeling that he is a master of the subject, that he is now prepared to teach others, and often even to show off to others his brilliant knowledge. The second method is the more difficult one, the less acceptable to the student, and often the more discouraging. The student who manages, however, to survive this type of training is the more humble in his approach to the unknown universe; he is more inclined to continue

the search for more information. This approach to the education of a scientist reminds me of the story told by Morris R. Cohen: "As a teacher," he said, "one must clear the ground of useless rubbish before one can begin to build." When reproached by one of his students for his destructive criticism, he replied: "You have heard the story of how Hercules cleaned the Aegean stables. He took all the dirt and manure out and left them clean. You ask me, 'What did he leave in their stead?' To this, I answer, 'Isn't it enough to have cleaned the stables?'" I am afraid that while this reply may have satisfied students in philosophy, it would hardly have done so for students of the experimental sciences, certainly not for the great majority of them.

An extreme of the second method of education is illustrated by the story told of Wilhelm Beijerinck. Upon entering the laboratory one morning, he found one of his students staining a bacterial preparation. When he asked the student the nature of the stain that he was using, the reply was: "Gentian violet." Beijerinck then proceeded with a question as to the chemical structure of the stain. The confused student pleaded ignorance. Beijerinck commented caustically that an investigator had no business to carry out research by the use of tools the nature of which was unknown to him.

What about the relation of the scientist to his environment? On the one hand, we have the concept of Emil Roux, a former director of the Pasteur Institute, who believed that a scientist should devote the whole of his life to science, should not possess a private or personal life, should not even marry; no wonder that he was often spoken of as "moine laïque" or "lay monk." On the other hand, we have the concept of the true biologist, who sees in all human lives a constant adjustment to the environment. This is well expressed by Raymond Pearl:

"Because a person, from however pure and noble mo-

tives, elects to be a worker in science he is not thereby absolved from the duties and privileges of being human. He must work out an adjustment between the claims upon his life by his science, a proverbially jealous and exacting mistress, and those of the rest of the world, including not only deans, committees, commissioners, directors, boards, foundations and other great cosmic elements, but also cooks, maids, nurses, children, and most important of all, his wife. If our graduate student, in whose behalf we are taking all this trouble, turns out to amount to much he will sooner or later receive offers for the purchase of his soul. Such offers will be made by those skilled in the traffic and they will be tempting. Shall we not be derelict in our job of helping our student to get his training for life if we do not furnish him some insight into what wisdom is available about the making of these necessary adjustments between scientific research and the rest of life?"

We are now faced with the problem of selecting a group of scientists who would come to this institute and who would labor to advance our knowledge of microbes. There are, of course, those who have already established themselves, who have already demonstrated their ability to contribute to this search for the unknown. There are, on the other hand, those who are still young and inexperienced, but who, given the proper opportunity, will develop into brilliant investigators. But how is one to select the latter? We can only think of a most delightful conversation that took place in 1813 between Sir Humphrey Davy and his friend Pepys: "Pepys, what am I to do? Here is a letter from a young man named Faraday. He has been attending my lectures, and wants me to give him employment at the Royal Institute—what can I do?" "Do?," said Pepys, "put him to wash bottles; if he refuses he is good for nothing." "No, no," replied Davy, "we must try him with something better than that."

Should one decide upon such promising young investigators, one should think at once of the admonition given by Charles Richet in his *Natural History of a Savant*: "Young man, if you would discover a new truth, do not seek to know what use will be made of it. Do not ask in what way medicine or commerce or industry may profit by the discovery; for if you do, you will discover nothing at all. You wish to find a solution to a problem that you consider important: tackle the problem without worrying about the consequences. Attack the question on its simplest side. Do not be stopped by the criticisms of journalists, hygienists, engineers, chemists, doctors. Let them talk. Go straight to the problem by the shortest road. Leave to practitioners the cumbersome care of consequences and industrial applications. *Veritas lucet ipsa per se*. Truth is sufficient unto itself."

One must not forget that the time when a Faraday could make a great discovery merely by using a piece of string and copper wire and working in a cellar has long passed. The modern chemical and biological laboratories require expensive equipment. The research problem frequently involves the collaboration of several investigators and certainly that of the senior investigator and his assistants, the teacher with his long and varied experience and the student who has come to be taught and guided and to benefit from such experience.

The question frequently arises as to how to distribute the credit for a new discovery, a new fact, a new observation. Some investigators will credit their assistants merely by footnotes on scientific papers; others will exert every effort to encourage their students, inspire them and stimulate them further to select research careers, and in doing so, may not only add their names to the scientific papers, but even place them first. In most cases, the results of the second practice are entirely satisfactory. Often, however, the consequences are most unexpected. How should one



strike a happy medium? Scientists are searchers for truth. But they are also human beings.

Finally a word must be said of the concept of "pure" or "basic" and of "applied" or "practical" research. We have been hearing a great deal about these lately. One is supposed to designate the study of fundamental principles underlying any natural phenomena, and the other the use for practical purposes of information thus gained. A German investigator who died recently is said to have commented: "I am dying happily with the knowledge that out of my 50 years of research not one of my discoveries has found any practical application." Personally, I prefer the concept of Pasteur, who emphasized that science and the applications of science are related to each other as are the fruits to the tree which bears them. In the words of Pasteur: "When one has reached the certainty of having discovered an important scientific fact, one experiences the greatest joy that can be felt by a human soul, and the thought that one has contributed to the honor of his country makes this joy even more profound."

It is my sincerest hope that while the workers in this institute will concern themselves with the search for truths basic to our knowledge of microbes, their nature, their occurrence, their manifold activities, and their role in natural processes, they will not overlook any potential utilization of facts thus obtained for improving our health, combating our natural enemies, increasing the productivity of our mills and our fields, and advancing other aspects of human welfare which will go to make the life of man easier, better, and happier.

The functions of this institute will comprise both research and teaching, largely on graduate and postdoctorate levels. The staff of the institute will offer courses in the various fields of microbiology to graduate students and will conduct seminars for graduate and postdoctorate students. These courses will also include supervision of vari-

ous research problems, in both the fundamental and the applied phases of the subject. The institute will serve as a gathering place for seminars and conferences on microbiological subjects. It will also serve as a depository of cultures of microorganisms of theoretical or practical importance.

In planning this institute, departmentalization has been avoided. It is our sincere hope that research programs will be flexible, investigators will be free to pursue their chosen fields without restriction, "team research" will be encouraged whenever possible, especially when "it grows out of genuine supplementation of contributions" and "when it serves the purpose of developing ideas" (Curt P. Richter). Opportunities will be given for younger men and women to obtain knowledge and guidance in their selected fields.

In concluding, I should like to repeat what I said two years ago, in connection with the laying of the cornerstone of this building:

This institute will devote its efforts to the study of the smallest forms of life, the microbes, wherever they are found and no matter what their activities may be. Let this institute serve as a center where scientists from all parts of the world may gather to work, to learn, and to teach. The halls of this institute are dedicated to the free pursuit of scientific knowledge for the benefit of all mankind.

# *From Dutch Settlements to the Rutgers Institute of Microbiology*

By ALBERT J. KLUYVER  
Professor of Microbiology,  
Technical University, Delft, Holland

It will be unnecessary to assure you that I felt greatly honored on receiving the invitation to address this distinguished audience on behalf of the microbiologists from abroad. But it was only natural that I also started pondering why this privilege was granted to me.

Fortunately, the letter of invitation contained the clue to this riddle, for mention was made of the close ties that from the very beginning have linked Rutgers University with the Netherlands.

To the Dutch ear the name Rutgers University immediately suggests that at some time there must have been an intimate relation between this institution and a man of Dutch descent. True, the great philanthropist Henry Rutgers, who in 1825 at the age of 80 succeeded in acquiring immortality by his, for those days, princely gift of \$5000

to Queen's College, was born in New York. But the name Rutgers—unpronounceable for the Anglo-Saxon tongue—leaves not the slightest doubt regarding the origin of his ancestors. Even nowadays descendants of the patrician family Rutgers enjoy great esteem in Holland.

Apart from the university's name, there is also the donation made in 1927 by the Holland Society of New York: the fine statue of William the Silent on the campus, which testifies to the fact that also in later years the university has been fully aware of the important part Dutch settlers played in its foundation. I need scarcely say that it gives me the greatest satisfaction to speak before the representatives of a university in which the Father of my Fatherland, this great champion of tolerance and defender of freedom, is held in such high esteem.

Both the university's name and the statue are, however, mere symbols of the good relations which of old have existed between the State University of New Jersey and the Netherlands. A more convincing testimonial to this bond can be found in the fine history of Rutgers College published in 1924 by your former president, William H. S. Demarest. The first chapters, dealing with the origin of the college, solidly document the fact that the initiative for its foundation was taken by settlers of Dutch descent, in close consultation with the Dutch Reformed Church. Demarest says, regarding these settlers, "The college is a child of their fine tradition, their zeal for education, their devotion to the faith, and of the compelling circumstances in their new American life."

I feel fortunate that I can quote from such an authoritative and unbiased source, since a perusal of the book has taught me that not all my fellow countrymen have under all conditions been favorably disposed to the idea of founding a new center of learning in the New World. Particularly, the Classis of the Dutch Reformed Church at Amsterdam seems to have opposed the project rather vigorously,

fear of the necessity of financial support from the homeland apparently being at least partly responsible.

But the indomitable spirit of the pioneers finally overcame all obstacles, and in 1766 Queen's College was chartered. In this first era, Dutch influence remained considerable. At that time the activities of the college were mainly restricted to those of a theological seminary, and many of the professors had studied in the Netherlands and received their degree of Doctor of Divinity at one of the Dutch universities. Probably the last one to do so was your great president, John H. Livingston. At the University of Utrecht, a bronze tablet erected in 1909 by the trustees, faculty, alumni, and friends of Rutgers College commemorates their appreciation for the part the university played in the cultivation of Livingston's scholarship. The inscription refers to the university as "*Mater Almae Matris Nostrae.*"

For this reason, I am particularly happy that the Rector Magnificus of the University of Utrecht has authorized me to offer to Rutgers University on this notable occasion the respectful and fraternal greetings and warmest congratulations of the Utrecht Academic Senate.

It seems appropriate now to review briefly the events that have led to our gathering at this moment at this particular spot. We all realize that the Institute we dedicate today is a direct outcome of the great heights that agricultural science has attained at Rutgers University. In turn, this is clearly connected with the high level of agriculture and horticulture in New Jersey, which earned it the epithet "Garden State."

I hope you can forgive a scientist for feeling the need to inquire into the causes of this particular aspect of the wealth of New Jersey, for despite the enormous industrial development that has taken place in recent years, "truck farming" is certainly still one of the pillars of the state's economy.

Again I was fortunate to be able to satisfy my curiosity by a study of the excellent article entitled "Agriculture in New Jersey," written by the former secretary of the College of Agriculture of Rutgers University, Dr. Carl R. Woodward, as part of the monograph: *New Jersey: A History*, published in 1930. The article certainly makes good reading, especially for a Dutchman!

At the risk of becoming guilty of nationalistic boasting, I cannot refrain from borrowing some data from this eminently reliable source. By 1630, several Dutch farmers had settled on the west bank of the Hudson River and established plantations or "boueries," as they were then called, a word which, strangely, still survives as the name of one of Manhattan's slums. A Dutchman by the name of Pauw obtained title to the greater part of the territory now known as Hudson County, at that time called "Pavonia," after him. He must have developed his holdings energetically and successfully, for in 1632 it was reported that the "boueries" on the west side of the river were in a prosperous condition, the settlers growing grain and raising cattle and poultry. Many of these settlements were, however, wiped out by the Indian massacres in 1643 and 1654, only to be revived in 1660, better precautions being taken against Indian raids.

With the passing of New Netherlands to English control, the Dutch moved inland along the river courses. Among these, special mention is made of the Raritan, and it seems likely that the very spot on which we are assembled once formed part of one of the farms that in 1680 were reported as "very fine and yielding well."

Woodward gives a long list of the vegetables, fruit, and flower plants that were imported in the seventeenth century by the settlers from the Netherlands and that proved to thrive exceedingly well in the fertile New Jersey soil. Woodward adds that "the Dutch were the first people to grow the European grains in the New World on a profit-

able commercial basis," and refers in this connection to wheat, rye, and barley. He finally states that to the Dutch goes credit also for the early introduction of the best foundation stock of horses and cattle. He further mentions the importation of hogs and numerous types of domestic fowl.

Altogether, I do not think I go too far in concluding that Dutch farming experience, and plants and animals of Dutch stock, have been mainly responsible for laying the foundations on which rests the well deserved fame of New Jersey as the Garden State.

This digression may find its justification in the fact that it assists in explaining exactly why New Jersey has been such a fertile soil for development of the agricultural sciences. This development can be said to have started in 1864 when the State of New Jersey assigned to Rutgers College the responsibilities and benefits of the Land-Grant Act of the Congress of the United States. Rutgers has responded nobly to this assignment. In 1939, Dr. Waksman, in his article entitled "Cook-Voorhees-Lipman: Contributions of Rutgers to Soil Science," paid warm tribute to the eminent scientists who earned a world reputation for the New Jersey Agricultural Experiment Station, the organization through which the Rutgers efforts in this field have been channeled since 1880. Moreover, the attractive booklet that appeared only three years ago on the occasion of the dedication of Lipman Hall testifies in a convincing way to the fundamental contributions to soil science made by Rutgers.

Suffice it here to mention a few facts that may be deemed to be directly responsible for today's ceremony. We must first go back to the foresight of Dr. Edward B. Voorhees, who as early as 1895 started investigations of soil microbiology and in 1901 established a separate Department of Soil Chemistry and Bacteriology, the first of its kind in the United States.

In view of the present-day emphasis on biochemical

studies in microbiology, it is worth mentioning that the first Nichols Gold Medal of the American Chemical Society was awarded to the bacteriologists Voorhees and Steel for their paper entitled "Studies in Denitrification."

The initiative taken by Voorhees was magnificently carried on by Dr. Jacob G. Lipman, who in 1911 succeeded him as director of the Experiment Station. Of the many great merits of Lipman, I mention only his clear recognition of the vastness of the domain in soil microbiology that was still waiting for scientific exploration. He made several valuable contributions to our knowledge of the microflora of the soil, and in numerous reviews and books did a great deal to stimulate interest in the field. In the outstanding journal *Soil Science*, which he founded and of which he was editor-in-chief until his death, he erected an imperishable monument to himself. Finally, he proved his great wisdom in the selection of collaborators worthy of the high reputation of the Experiment Station. Of these I will mention only two: Dr. Selman A. Waksman, who entered the station in 1918 and who succeeded Lipman after his death in 1939, and Dr. Robert L. Starkey, who joined his staff in 1926.

Both scientists are here in our midst. I need not discuss the highly important work they have done that has made the New Jersey Agricultural Experiment Station the mecca of soil microbiologists from all over the world. Characteristic of the way in which the problems of soil microbiology have been attacked here is the purely scientific spirit in which the investigations have been conducted. Increased knowledge of the microbial world in its divergent manifestations has always been the leading principle.

Studies of this type usually fail to impress the general public, but as the representative of so many microbiologists assembled here, I think I may and should testify that our admiration for Dr. Waksman as a scientist dates back



to a time long before the words "antibiotics" and "streptomycin" were coined.

Pasteur once remarked: "Le hasard ne favorise que les âmes préparées" (chance favors only those who are prepared). For insiders it is quite clear that this dictum also applies to the great discovery of streptomycin. If Dr. Waksman had not made such extensive studies on the actinomycetes as long ago as the 1910's, in a period in which almost no one had any interest in this microbial group, and if Dr. Waksman had not so thoroughly investigated the fate of certain microbes in the soil, thus throwing a clear light on the chemical warfare in which so many microbial types are continuously engaged, this ceremonial meeting would never have been held.

For this reason, the microbiologists of the world wish to honor Dr. Waksman above all as the disinterested man of science who for almost forty years has made so many important contributions to pure microbiology. But in addition, we do not lack in admiration for the great ingenuity and the exemplary perseverance with which he pursued his studies as soon as it became clear that microbial antagonism could be directly applied to the alleviation of the sufferings of mankind. Nor can we fail to express our deep appreciation for the fact that he has directed certain material consequences of his work in such a way that they resulted in the splendid institute we dedicate today.

The prospects of the new institute seem bright. It has been designed and equipped by a man who won his spurs in the field. Moreover, the same man will be its first director and determine its course. Anyone who has read the recent article on the man of sixty-five in the British medical journal *Lancet* will agree with its general conclusion: "Do not put him on the shelf." On the contrary, realizing how greatly our standard of old age has changed in recent decades, we are certain that his accumulated wisdom will

prove to be an invaluable asset, and to his guidance we all look forward with the greatest confidence.

In the name of the microbiologists of all nations, I herewith offer to the new Institute of Microbiology our very best wishes for its lasting prosperity. The institute has been born of microbial antagonism. May it forever be a symbol of human symbiosis, and may it flourish to the benefit of our beloved science and of mankind as a whole.

Mr. President, I extend to you our warmest congratulations on the scientifically highly important addition that today is made to your already renowned university.

In concluding, I may perhaps formulate one more wish. It was your former President Livingston, who, inspired by the motto of the University of Utrecht, *Sol justitiae illustra nos*, chose as the motto of Rutgers University, *Sol justitiae et occidentem illustra*. May the time not be far off when the sun of righteousness will shine equally brightly upon both Occident and Orient. But regardless of developments elsewhere, may it forever illuminate Rutgers University.











