RECENT ADVANCES IN OUR KNOWLEDGE OF THE PHYSIOLOGY OF MICROÖRGANISMS[1]

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About a century ago Pasteur started to develop his thesis that there are a large number of characteristic decomposition processes of organic materials which are brought about by the activities of various microörganisms. As good examples may serve the conversion of sugars to alcohol and carbon dioxide, to lactic acid, to butyric and other fatty acids and gases; the oxidation of ethyl alcohol to acetic acid, etc. For each of these processes a certain microbe or a group of closely related organisms was held responsible.

Most of Pasteur's studies in this field were carried out with cultures which nowadays no one would be willing to consider "pure" in the strict sense of the word. This did not materially affect the development of Pasteur's thesis which has gradually become one of the foundations of our present-day science. That, even with impure cultures, Pasteur could arrive at this fundamental concept is due in part, but only in part, to his keen power of observation. Nevertheless, an important adjunct has unquestionably been the fact that he used to a large extent simple and logically composed culture media for his experiments. This resulted in the rapid development of a microflora which, in many cases, was restricted to a few easily recognizable types.

Pasteur also anticipated that microörganisms play a significant rôle in human welfare, especially as causative agents of diseases. The careful study of these phenomena, soon initiated, proved of such vital importance that in a relatively short time tremendous strides were made in the diagnosis, cure and control of disease. So spectacular were the results that it is fully understandable how this aspect of the activities of microörganisms rapidly attracted nearly all the attention, of students and public alike. To a great majority the study of these creatures became the equivalent of the study of diseases, their cause and cure.

Nevertheless, there were a few scientists, like de Bary, Cohn, Winogradsky, Beijerinck, who, by their investigations of the general aspects of the microbes themselves, kept alive an interest in the more fundamental problems of biology. In recent years it has become clear that this has been fortunate; more and more the evidence points to the benefits that can be derived from the use of these "simple" living beings for the study of the basic problems of life.[2]

At present there is a distinct tendency to recognize this, and to attempt an

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[2] *Cf.*, also, Otto Rahn's statement: "While practically all the discussion in the book refers to bacteria, the principles developed reach beyond the domain of bacteriology, and apply to biology generally. More than that, I believe that some of the principles of biology can be found and studied *only* with the simplest forms of life, and that general physiology has much to learn from the physiology of bacteria." (1, p. viii).

amalgamation of various interests, a new integration of knowledge and viewpoints. The inclusion in the program of a general meeting of our Society of a broad paper on the physiology of microörganisms before a joint session is a definite sign of such a drift.

To the speaker who, for many years, has realized and advocated the advantages of "General Microbiology" this is, of course, gratifying. It involves, however, the difficulty of preparing a presentation which, on the one hand, is simple enough to make the problems intelligible to a general audience, and on the other, includes enough of interest to the various "specialists". In spite of the temptation not to disappoint the latter group, I have decided to concentrate on the former aspect.

As previously stated, Pasteur's approach to the culture of microörganisms was eminently rational. The advent of "medical bacteriology" gradually led to an unfortunate change in this respect. I do not mean to imply that it was irrational to use tissue fluids, blood fractions, meat extracts, physiological salt solutions, and the like for the culture of bacteria occurring in body tissues. On the contrary, the choice of such ingredients appears entirely logical. But the fact is that media of this type could be used satisfactorily also for growing the great majority of non-pathogenic organisms. And this resulted in the adoption of such complex materials as "standard media" for general use. Thus the culture methods became stereotyped, entailing the regrettable consequence that one of the more important problems of microbial physiology, that of the nutrition of microörganisms, was not even recognized as such by most bacteriologists. It is true that there were isolated instances which clearly demonstrated an awareness of the fundamental principles of nutrition. Nevertheless, it was not until recently that microbial nutrition began to be studied as a legitimate and significant problem. Much of our present knowledge derives more immediately from the work of Lwoff (2, 3) and Knight (4).

Just what is it that microbes need as food, in order to enable them to grow, to reproduce themselves? Since not a single case is known in which these living creatures accomplish a transmutation of elements, it is obvious that all elements which are part of the living cell must be present in their environment if they shall be in a position to grow. But the form in which these elements are useful for the building of new cells differs widely for various organisms.

With the exception of carbon and nitrogen, all such elements can be satisfactorily supplied as inorganic salts. The great diversity occurs with respect to the carbon and nitrogen nutrition. While a great many microörganisms can manufacture all their cell constituents from nitrates or ammonium salts and a single simple carbon compound, such as ethanol, acetic or lactic acid, or sugar, numerous others require additional and more complex substances before they can be made to grow. Some simple considerations will help in clarifying the situation.

As far as has been ascertained, all living organisms are composed of water, carbohydrates, proteins, fats and salts as the quantitatively predominant constituents. In addition they must, in order to function properly, also contain enzymes, those "biological catalysts" which have been made responsible for most biological activities which reveal themselves in chemical transformations of various sorts, generally referred to collectively as "metabolism". It is then obvious that an organism which can grow, for example, in an inorganic salt solution with ammonium acetate or lactate, must also be able to elaborate all its proteins, carbohydrates, fats, as well as enzymes, from these ingredients. On the other hand, an organism which cannot synthesize an essential protein constituent, such as tryptophane, obviously cannot produce new cells unless it be simultaneously supplied with either tryptophane itself or with a substance from which it can produce tryptophane.

This is quite elementary, as are many problems when properly stated. The great importance of so viewing the topic lies in the immediate clarification of the true nature of the nutritional problems. It was Lwoff who, in 1936, first clearly stated that any substance which an organism cannot synthesize, and yet needs for its developments, constitutes for that organism a "growth factor" (5). This statement appears eminently logical, and, in its ultimate consequences, unavoidable. As a rigorous definition of the term "growth factor" it is, however, not generally adopted; there are various other attempts at defining "growth factors", usually implying that these should be substances of organic nature and required in minute amounts. No matter what special definition or terminology be applied, the concept contained in Lwoff's statement makes it possible to draw some important inferences which have already proved extremely fruitful, as may appear from the following recent developments.

The decisive need for certain specific organic molecules in the nutrition of a microbe implies, on the basis of the above mentioned concept, *a*, that such compounds cannot be manufactured by the organism in question from other substances and *b*, that they must play a fundamental rôle in the well-being of the cell which, without a definite supply of these materials, cannot function normally. Where this mode of reasoning is applied to those growth factors needed in minute amounts, it is, furthermore, necessary to conclude that only a relatively small number of molecules per cell is sufficient to satisfy its needs. This, in turn, leads to the inference that such a substance, the qualitative requirement for which appears so much out of proportion with its absolute importance, must fulfill an extremely active rôle. In this manner we are led to search for a direct connection between this group of growth factors and highly active cell constituents—that is, enzymes. If the particular organic molecules were needed for the manufacture of enzymes, then both the very small amounts required, and the vital necessity would be accounted for.

Here it may be recalled that the first chemically recognized enzyme constituents, riboflavin, thiamin, and nicotinic acid or its amide, were almost simultaneously identified as specific growth factors for certain microörganisms. And these are also substances which have been known as vitamins for the higher animals.

There are a number of cases where a fundamental connection if not identity between such growth factors for microörganisms and typical vitamins has been revealed. In fact, it is an easily defensible thesis that all vitamins for metazoa will have their counterparts among the microbial growth factors. This, of course, strengthens the view that, in its basic features, the metabolism of all living organisms is fundamentally the same. And thus studies on growth factors for bacteria, fungi, and protozoa are of importance not only for the acquisition of a better understanding of the nutritional problems of microörganisms *per se*, but also for the prospect of the discovery of as yet unknown vitamins. Furthermore, those microbes which display an absolute requirement for specific vitamins have already proved extremely useful and inexpensive tools for the purpose of vitamin assays (6).

One further implication of the interrelations between certain growth factors, vitamins, and enzymes is that those microörganisms which can develop in an environment devoid of one or more of such substances must be able to synthesize them from other foodstuffs. Wherever this has been tested experimentally, it has been found to be true, as has also the conversion of these materials into enzymes in the relatively few cases in which such demonstration has been practicable.

In the foregoing paragraphs a deliberate attempt has been made to discuss the nature and function of specific growth factors in the most elementary sense. Not always are the experimental results quite as clear-cut or simple as those here considered. It occurs not infrequently that the development of a microbe, while not strictly dependent upon the availability of some special substance, can be greatly furthered by its presence. The common practice of referring to this type of compound as "growth-promoting factors" has tended to obscure the fundamental concept of the nutritional problem. This is, however, not necessary because results of this kind can be satisfactorily interpreted in line with the earlier developed hypothesis. It may readily be assumed that an organism which shows such a response is intrinsically capable of performing the synthesis of the related cell constituent from other nutrients, but that this occurs at so low a rate that the over-all phenomenon of growth becomes limited by this particular synthetic process. Hence the presence in the medium of preformed molecules of the compound in question eliminates the necessity for the slow synthesis and consequently can result in an acceleration of growth, now primarily restricted by synthetic processes which take place at a considerably greater rate.

While this phase of the study of microbial nutrition has thus yielded results and concepts which seem clearly to establish the relation between certain kinds of growth factors and vitamins, it cannot yet be asserted that the connection between these substances and special enzymes has been demonstrated equally satisfactorily. This, however, is undoubtedly the result of a still very limited knowledge of the specific chemical nature of the enzymes; the pursuit of growth factor studies has considerably exceeded that of the chemical composition of enzyme systems. The chief reason for this lies in the difficulties connected with the purification of enzymes. Even the preparation of crude but active solutions has, in many cases, proved far from simple.

Nevertheless, advances have been made in this respect, in part due to the introduction of new techniques. Among these the grinding mill of the English workers (7) and the powdered glass technique of Werkman *et al.* (8) are especially worthy of note. It becomes increasingly clear that a satisfactory picture of metabolism must entail a more penetrating comprehension of the chemical nature of these processes. And in view of what has been achieved in a few isolated cases, it is not too much to hope that this will ultimately lead to a fine resolution of the over-all changes of foodstuffs to excretory products and production of cell constituents into a series of simple steps, each proceeding under the influence of its particular and special enzyme system.

The few general principles in this field which have been developed in the past 25 years, and which have proved so eminently fruitful in guiding our future progress, have above all comprised the concept that any biochemical process consists of a more or less extended series of step-reactions, proceeding in order, and each one step representing a simple, chemically intelligible reaction. If the primary substrate consists of complex carbohydrates, oils or fats, or proteins, it was considered probable that these would undergo a preliminary breakdown by hydrolysis. The split products would then be subject to a series of further breakdown reactions the nature of which is in essence a hydrogen transfer. Hereby the original substrate is either oxidized, or it may be split into smaller units, while other substances or some of the split products are simultaneously reduced.

Various objections have been raised especially against the assumption of a preliminary hydrolysis of di- and polysaccharides. A number of experiments have indicated the existence of microörganisms which are able to decompose some polysaccharides but which apparently do not attack the constituent hexose units. A careful study of such cases has, without exception, shown that the interpretation of the experimental results was at fault. One of the most persistent claims, pertaining to the decomposition of cellulose by a group of bacteria unable to utilize glucose, was resolved recently by Stanier (9) who emphasized once again the long known but often forgotten production of toxic products during the heat-sterilization of glucose solutions. There is at present not a single authenticated instance on record of microörganisms which can attack some polysaccharide but not its hydrolytic products.

There are, however, some well-verified examples of organisms decomposing di- and polysaccharides faster than the simple hexose constituents. Clearly this could not be so if the supposed hydrolyses initiate the decomposition of the polymers. The elucidation of this situation has resulted from the application of the discovery, by Cori (10), that muscle tissue depolymerizes glycogen with the production not of glucose but of glucose-1-phosphate, the now famous Cori-ester. The greatest importance of this discovery lies in the fact that this type of reaction is enzymatically controlled, and reversible, thus making possible the enzymatic synthesis of glycogen or starch from glucose-1-phosphate. This concept was applied by Doudoroff to the decomposition of sucrose by *Pseudomonas saccharophila*, which proceeds considerably faster than that of glucose, fructose, or invert sugar (11). A spectacular but logical outcome of this investigation has been the experimental demonstration of an enzymatic synthesis of sucrose, not from glucose and fructose, but from Cori-ester and fructose, again as a completely reversible process (12).

While it would be premature to assert definitively that all decompositions of di- and polysaccharides proceed by way of such "phosphorolyses" rather than hydrolyses, it appears, nevertheless, as a most logical and attractive working hypothesis. This manner of depolymerization leads directly to products which function as the first intermediate stages in the gradual breakdown of the simple sugars.

It should be emphasized that these recent developments in an understanding of carbohydrate breakdown imply far more than the replacement of a theory, gradually shown to be inadequate, by one which is at present more satisfactory; they also pave the way towards a deeper insight into the synthetic processes in metabolism.

For a long time physiologists have been more or less satisfied with the concept that metabolism consists of two fundamentally opposite processes: catabolism and anabolism, or breakdown and synthesis. These two phases were considered as energetically coupled; the synthetic processes, requiring energy, were made possible by the occurrence of catabolic reactions in which energy is liberated.

But the successful interpretation of the more intimate mechanism of catabolic processes has inevitably led to the desire to approach biological syntheses in a similar manner, and to comprehend the precise chemical nature of such reactions. It is obvious that the conversion of lactate and ammonia into carbohydrates, proteins, fats, nuclear materials, enzymes, etc., is ultimately chemistry in the same sense as is the conversion of sugar to alcohol and carbon dioxide, or to lactic acid. The only real difference is that the former conversions are far more complicated. Now, the first examples of an enzymatic synthesis of carbohydrate show an important principle: it is not the hexose itself but a product, arising therefrom in the course of the gradual breakdown, which functions as the immediate raw material from which a spontaneous, enzymatic synthesis can proceed.

A very similar situation has been known for some time in the case of the synthesis of amino acids. This has been achieved by the addition of ammonia to a keto acid or to an unsaturated acid with the subsequent reduction of the imino acid. Both these reactions have been recognized as reversible and enzyme-controlled, (13). Recent evidence to the same effect has come from the studies of Bonner *et al.*, which have shown that mutant strains of *Neurospora*, unable to synthesize leucine, isoleucine, and valine from glucose and ammonium salts can do so if supplied with the keto-acid analogs of these amino acids (14).

We are, therefore, in possession of a number—a very small number, it is true— of examples which show that synthetic reactions, polymerizations as well as the formation of new linkages, are comprehensible as enzyme-controlled, simple chemical processes. Also in this category belongs the synthesis of four-carbon compounds from three-carbon compounds by the addition of carbon dioxide to a three-carbon molecule, discovered by Wood and Werkman (15). This type of reaction is now recognized as of extremely wide-spread occurrence (16). One important aspect of these syntheses must again be stressed: the initial components for such reactions comprise in part not the original food constituents but conversion products thereof. And this group of products, *e.g.* Cori-ester, keto acids, etc., arise generally and readily during the gradual breakdown of the original substrate.

Following this trend of thought, it becomes rational to look upon the biological syntheses as the result of series of consecutive step reactions for which one or more of the reactants are furnished immediately by catabolic processes inasmuch as they represent intermediate products in the chain of individual steps comprising the breakdown of the substrate.

This is also the place to call attention to the eminently important studies which have led Lipmann and others to a new concept connected with the gradual degradation of oxidizable materials by dehydrogenations (17, 18). Formerly it was considered probable that, whenever a double bond originated by dehydrogenations—as, for example, in the formation of an aldehyde, keto acid, or unsaturated fatty acid—the addition of water as H and OH to the double bond would precede a further dehydrogenation. It now appears likely that, in analogy with the addition of phosphate during phosphorolysis of polysaccharides, also here not H_2O but phosphate is added. The subsequent oxidation of such phosphorylated products then leads to the formation of curiously unstable, that is highly reactive, substances from which a number of syntheses may be expected which it would be impossible to achieve with the phosphate-free molecules. And it is of great significance that Vogler and co-workers have shown that even during the oxidation of an inorganic substrate, *viz.* sulfur, by the autotrophic bacterium *Thiobacillus thiooxidans* such labile, active organic phosphate compounds arise, especially since it has further emerged that these substances are intimately linked with the synthesis of cell materials from carbon dioxide (19). That it is very probable that also in the photosynthetic carbon dioxide assimilation such phosphorylated substances participate can only be mentioned in passing (see, for example, 20); suffice it here to emphasize the very general importance of phosphorylated organic compounds for the problem of biological syntheses.

Hence it appears that a profound study of the steps involved in the breakdown reactions will do more than yield a better insight into the mechanism of just these processes. It would furnish ever more complete information as to the exact chemical nature of the various intermediate products, and thus also tend to reveal the steps by which biological syntheses proceed. It is particularly in this sense that studies such as Foster's on the microbial decomposition of riboflavin must be understood; they aim ultimately at the elucidation of the mechanism of its biological synthesis (21).

But it is not only a study of catabolic reactions which can supply important information concerning the mechanism of anabolic reactions. Other approaches are possible, and one, in particular, has contributed some beautifully clear results.

In principle it rests on the following consideration. An organism may be unable to develop in a simple medium because it lacks the ability to synthesize a specific component of its normal cellular constituents. When, therefore, the organism is inoculated into the simple medium enriched with this substance, growth will occur. By supplying, instead of the compound itself, such materials as may be deemed probable precursors in its biosynthesis, the experimenter can deduce from the growth response of the organism which substance or substances are stages in the elaboration of the specific cell constituent. It will be clear that the previously mentioned demonstration of the formation of certain enzymes from vitamins is a case in point. Here, however, the details of the biosynthesis are still obscure. Much better examples are furnished by the synthesis of thiamin from the corresponding thiazole and pyrimidine moieties, and of biotin from pimelic acid (3, 22).

Experimental demonstrations of this kind depend upon the availability of organisms known to lack a specific synthetic function, and the literature pertaining to this subject contains a number of examples of such organisms. But until recently this experimental material was restricted to chance isolations. The purposeful production by Beadle and Tatum of special mutant strains of microörganisms, especially of *Neurospora crassa*, by x-ray or ultraviolet irradiation of vegetative cells or spores, has brought about a most auspicious change in this regard (23). Their studies have made available an intensely interesting collection of cultures the members of which are characterized by the inability to synthesize one of a variety of cell constituents. With this material the biosynthesis of arginine from citrulline, and of the latter from ornithine has been shown conclusively (24). The elucidation, by Tatum and Bonner, that the biosynthesis of tryptophane does not proceed from the corresponding keto acid and ammonia, but from a direct junction of indole and serine has also been made possible by the use of these mutants (25). This last investigation is therefore so important because it reveals a new type of synthetic reaction in biological systems which is likely to have a far more general significance for an understanding of the mode of formation of alkyl-substituted aromatic compounds.

The importance of microörganisms for the study of biochemical problems cannot be overemphasized. It is among them that one meets with the almost unlimited diversity of biochemical peculiarities which offers the greatest opportunity for selecting the ideal material, and in its simplest form. It is unnecessary to go into details; it may suffice to mention the existence of organisms which cause the gradual breakdown of some substrate in such clear-cut stages that any one can be isolated and studied separately, as well as of organisms which lack the capacity for synthesizing one single, essential cell constituent from the components of a simple medium, and with which, therefore, precursor studies can so effectively be initiated.

Science is characterized by both a gradual approach and integration, and by a constant attempt at re-defining "ultimate causes"; or, in other words, by a continued search for a more and more detailed explanation.

Where once "miasma" served as an adequate designation of the cause of certain diseases, it was, after the great work of Pasteur and Koch, more satisfactorily replaced by "specific microörganisms". Later a still deeper penetration became possible. While mediately the bacterium, fungus, or protozoan still remained the "cause", it was the production of a toxin, the destruction of erythrocytes, etc., which more immediately was held responsible.

In the biological sciences, it is one of the present trends to view the events of life in terms of comprehensible chemical processes. The studies of vitamins, hormones, chemotherapy, the newer developments in experimental embryology and genetics, these all show conclusively how great the advance has been. And to the student of microörganisms, it is gratifying to note how impressive a rôle these minute living creatures have played in those developments.

What the future may bring, "who dares predict?" At present it seems inevitable that for a while the field of microbiology will draw an increasing number of students who will devote themselves to the investigation of a number of the fundamental aspects presented by this vast material with its inexhaustible potentialities. And it is likely that there will always be a few, at least, who will continue to do so after the majority has lost sight of the basic problems which the microbes will always offer.

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