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The Integration of Research on the Nutrition and Metabolism of Micro-organisms

The Inaugural Marjory Stephenson Memorial Lecture

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The Society could have done me no greater honour than to invite me to give the first of the memorial lectures to my old chief and teacher. She was one of the pioneers in her branch of microbiology, and in this first lecture it might be appropriate to examine a little the philosophy she reached after her lifetime of work in the field, especially in relation to the help it has brought and will bring to her successors.

Marjory Stephenson played a very active part in the foundation of our Society; this was because she had always been convinced that the most rapid development of microbiology depended on the closest liaison between those interested in the more biological and biochemical aspects. It is the biological field which sets the problems which she was convinced the biochemical approach could help to solve. At the same time she was just as sure that biochemistry owed an equal debt to microbiology: her address at the Congress on the occasion of the fiftieth anniversary of the death of Pasteur was indeed devoted to this theme (Stephenson, 1946).

At the inaugural meeting of our Society on 16 February 1945 Marjory Stephenson summarized her own experience and thought and drew for us a simple overall picture of the various methods of approach to research in microbiology, and especially chemical microbiology. I have always felt this to be one of those clear-sighted and simple statements of general principles which remain indefinitely helpful and become, so to speak, part of the common-law of a science. Unfortunately, we have no published account of this actually from her own pen, but there is a summary in a report of the meeting (Anonymous, 1945: see also Elsdon & Pirie, 1949; Woods, 1950*a*). She defined five levels at which research was carried out both from the biological and biochemical aspects. It was particularly emphasized that no one level was more important than another: they represent a spectrum rather than a ladder. Let us examine these levels (Fig. 1) briefly from the point of view of chemical microbiology in particular.

Perhaps the ultimate problem is to explain in biochemical terms the life of microbes in the mixed associations in which they usually exist in nature. It is unlikely that we shall dig very deep at this level until we know more about the precise chemical nature of the environment, the number and nature of the organisms present, and their biochemical potentialities: all these factors are likely to be changing constantly.

The first stage of simplification is, of course, restriction of the microbial population to a single species. The environment remains complex and undefined chemically. Here we hope to find out, from a comparison of the initial and spent medium, something of the overall metabolic activities of the microbe. It is of course at this level too that the great bulk of biological investigation takes place.

	Mixed cultures: complex 'natural' media [Organisms in natural environment]	Growing cultures
	Pure cultures: complex undefined media [General microbiology: overall metabolic picture]	
	Pure cultures: chemically defined media [Nutrition: biochemical mutants]	
	Cell suspensions (pure cultures): defined substrates [Overall products: intermediary metabolism]	Viable: usually no growth
	Cell preparations: defined substrates (a) 'Complete'—mixtures of enzyme systems (b) 'Fractions'—multienzyme systems (c) 'Purified'—isolated enzymes [Further detail of individual reactions]	Non-viable

Fig. 1. Levels of investigation in chemical microbiology.

At the next level the environment is simplified. We endeavour to establish in chemical terms the minimum requirements for optimum growth. This is the branch of our work usually called 'nutrition'.

Further simplification of the environment in order to study the action of a microbe on a single or relatively few substrates usually (though not always) results in conditions in which growth cannot occur. In order to obtain sufficient material, and to define the conditions more rigidly, we normally employ washed suspensions of cells harvested from a suitable growth medium. At this level we find out the overall products of action on defined substrates and, by traditional biochemical techniques, hope to begin breaking down metabolic processes into their individual steps.

The detailed analysis of a metabolic pathway must finally require the study of individual reactions as catalysed by individual enzyme systems. This is likely to yield most information if the enzyme can be extracted from the cell and purified. Furthermore, certain substances, which may be formed as intermediates within the cell and further metabolized therein, do not gain access to the cell when supplied externally; they can only be studied (if the enzymes survive) by non-viable cell preparations in which permeability barriers have been destroyed.

There is, of course, no clear-cut separation of these different levels; one can always see intermediate stages. At one end of the spectrum we are in closest relation to purely biological matters, and it is here that many of our problems are set. At the other end we become almost pure biochemists, and it is here

that we must not lose sight of our main objectives and regard micro-organisms simply as a convenient biochemical material.

Marjory Stephenson emphasized the importance of tackling a given problem at as many of these levels as possible. Research at one level may frequently come to a stop until new facts and new ideas become available from research at a different level. Put in a different way, these methods of approach represent the *tactics* of our attack upon a problem. The grand *strategy* is a co-ordinated attack on several fronts, shifting the main weight from one front to another as the results dictate and according to the availability of our experimental armament.

Apart from experimental convenience there are equally cogent reasons to subject results obtained at one level to rigorous test at others. To take one example only, it is clear that the work on adaptation and adaptative enzyme formation emphasizes the ability of the cell or culture to change both qualitatively and quantitatively its overall enzymic constitution.* The activities of washed suspensions of cells harvested at one particular stage of growth (usually towards the end in order to obtain enough cells) give us only a cross-section of the biochemical potentiality at that time. What we need eventually is an integration of all the metabolic events since the time of inoculation. This is provided by work with growing cultures but is more difficult to analyse; we must at least therefore try to discover, by reference to work at this level, whether the reactions we find with cell suspensions are indeed important for the growth of a culture. The adaptive powers of micro-organisms, though providing us with a valuable weapon in our experimental armament, do also complicate matters by adding, so to speak, a fourth dimension to our system of microbe, substrates and time.

For the remainder of this lecture I want to try to illustrate in particular how the integration of work at the several different levels covering nutrition and metabolism has advanced our knowledge of the synthetic mechanisms of the cell. Since co-ordinated biosynthesis is at its optimum in a growing culture it is natural to hope that work with such systems may provide valuable clues.

In attempting to give a broad outline, it is inevitable that I shall piece together so many individual contributions that it will be impossible to give proper acknowledgment to all those whose work is implicated. I am sure that for the purpose of doing honour to Marjory Stephenson those concerned will not mind, if, without permission, I nominate myself as their spokesman. Time will not permit me to recapitulate how these ideas grew up and proved their value. Also, since most members of the Society are not professional biochemists, I will try to set them out first in non-chemical terms, and then illustrate them with, I fear, woefully few concrete examples of their application.

* See, for example, the contributions to the Symposium on Adaptation in Micro-organisms (various authors, 1953) held at this meeting of the Society.

Pathways of biosynthesis

Let us consider a number of pathways leading to substances such as C, D and Z (Fig. 2) which are known essential metabolites necessary for the formation of new cell material. The information which we seek is (a) the chemical nature of the primary intermediates such as A, B, X, Y and so on, and (b) the properties of the enzyme systems catalysing individual steps such as $B \rightarrow C$ and $X \rightarrow Y$. The available methods of approach through studies of nutrition all depend really on finding what substances are needed to restore growth when there is some biochemical lesion, either natural or induced, which results in a failure at one step of a sequence of reactions.

Fig. 2. Scheme illustrating some different methods of experimental approach used in studies of biosynthesis. The symbols are explained in the text.

Types of metabolic lesion

Requirement for growth factors. The very fact that a number of organisms are unable to grow unless certain essential growth factors are provided is in itself a proof of failure to synthesize these substances and of the existence of a metabolic lesion compared with competent organisms. Such growth factors are of two main types: (a) substances incorporated into main cell constituents, e.g. amino-acids, and (b) substances of ultimate catalytic function such as members of the vitamin B group. The function of the latter, whenever it has been determined, has proved to be as part of the structure of coenzymes or prosthetic groups of important enzyme systems of the cell. This possibility may now be adopted as a working hypothesis in the case of new growth factors of unknown function.

Apart from organisms normally requiring growth factors, it is possible to obtain mutants (by irradiation, treatment with certain chemicals, etc.) which have lost the ability to synthesize particular essential metabolites. The

failure may be due in individual cases (including organisms with natural growth-factor requirements) to (a) the absence of an enzyme protein, (b) an enzyme with altered properties functional only under changed growth conditions, (c) the presence of an inhibitory substance, or (d) a failure to produce a coenzyme or prosthetic group.

Induction by substances similar in chemical structure to essential metabolites. Substances of similar chemical structure to a growth factor or intermediate (e.g. A^1 to A, F^1 to F in Fig. 2) often prevent the utilization of a metabolite by combining with and competing for the enzyme concerned. This in effect induces a lesion of the same type as that already discussed. Inhibition is overcome ideally (a) competitively by the metabolite itself, and (b) by the product of the inhibited reaction irrespective of the concentration of the inhibitor.

Failure to produce a coenzyme. If the growth factor is of the catalytic type (F), its absence will result in a failure to produce the coenzyme form (CoE, Fig. 2), and thus again a failure to carry out a particular enzyme reaction. Restriction of F may be attained either by omitting it from the culture medium, or by adding a chemical analogue of F which specifically inhibits its utilization. Growth may be restored either by adding the coenzyme itself or an intermediate in its formation (i.e. substances of the same chemical type as F), or by adding the product (C) of the inhibited reaction (i.e. a substance probably of dissimilar chemical type to F).

If the coenzyme is required, as is often the case, for two or more enzyme systems, then all the products (i.e. C and Z) will have to be added to restore growth. Addition of C alone would be expected only to reduce the amount of F required, but not to abolish the requirement.

Nature of intermediates

'Replacement' experiments. Suppose that we have a series of organisms in which metabolic lesions of this general type are present, or have been induced, with the result that all the organisms require the same factor (say Z) for growth. It is unlikely that all will have failed at precisely the same step in the synthesis of Z; some may fail to convert W to X, others X to Y and so on, i.e. the lesions are all within one synthetic pathway but at different points. Then with some organisms growth would be restored by Z only; with others by Y or Z but not W or X; with still others by X, Y or Z but not W. If, therefore, we test the ability of a number of possible intermediates to replace Z for growth, we should, and often do, obtain valuable information as to the course of the synthesis.

This approach is obviously more satisfactory when we can use the same species of organism throughout; this can be done in two ways. First, we may use the biochemical mutants; a number of strains with independent mutations leading to loss of ability to synthesize a particular metabolite are often available. If the test organism has a sexual phase in the life cycle, it is also usually possible to determine which of the mutants differ from one another genetically and are likely to have failed at different steps of the synthesis. Secondly, we

could also get results with a single species by using the inhibitory analogue technique for inducing failure to produce necessary coenzymes for different steps in the sequence of reactions; this approach has been little used so far.

Excretion or accumulation of intermediates. One difficulty of all these methods of approach is to decide, other than by intelligent chemical guesswork, which substances to try as possible intermediates. But the organism itself often provides valuable aid.

In all the types of metabolic lesion which have just been discussed it may happen that, when the final metabolite is provided so that the organism can grow, the intermediate which cannot be metabolized further continues to be synthesized; this intermediate (or a secondary product derived from it) accumulates in the cell and is often excreted into the culture medium. The isolation of such material has sometimes led to the identification of quite unsuspected intermediates. Such accumulations have been found frequently with biochemical mutants (e.g. W and X with mutants m_2 and m_3 in the scheme of Fig. 2); it has also occurred (e.g. B) when a reaction is prevented by the functional lack of a coenzyme, and again when the utilization of an intermediate (A) is prevented by an analogue (A^1).

That such excretion is occurring may be first indicated by the ability of the metabolic products of one strain to support the growth of another with a metabolic lesion at an earlier stage; thus in the scheme of Fig. 2 the mutant m_2 excreting W would support the growth of mutant m_1 which is blocked at the previous stage.

Simultaneous adaptation

A further method of detecting intermediates rests on the abolition of an existing lesion rather than the creation of a new one. Suppose that an organism does not normally metabolize N (Fig. 2), but forms an adaptive enzyme for doing so when N is present, the ultimate product being Q. Then it follows that, unless the organism normally has the power to convert O to P and P to Q, then adaptation to metabolism of N must also have caused adaptation to O and P. This can be tested with appropriate controls. The technique has been developed particularly by Stanier for the study of catabolic pathways, but it has not yet been possible to use it much in studies of biosynthesis.

Necessity for supporting evidence

The interpretation of results obtained by these various methods of approach is unfortunately rarely quite so simple and clear-cut as has been indicated in the formalized examples given. This makes it especially important that such results should be regarded as a signpost to future work and not as a proof of the mechanisms indicated.* Furthermore, since all synthetic reactions must require at least two substrates (e.g. Y and Q giving rise to Z), information about the other component may often still be lacking.

* Mitchell (1953) has recently particularly emphasized this point in connexion with work with biochemical mutants.

We must therefore seek supporting evidence at other levels of investigation, and the use of cell suspensions for studying the metabolism of defined substrates is clearly indicated.

Metabolism of cells deficient in a growth factor. Cell suspensions or tissues deficient in a given vitamin or growth factor have long been employed in attempts to locate the metabolic function of such metabolites. The technique was first used by Lwoff (1934) in the case of micro-organisms and by Peters & Thompson (1934) with animal tissues. The cells are harvested from media containing only sub-optimal concentrations of the growth factor and their metabolism compared in detail with that of normal cells. Any difference found may indicate reactions in which the factor has a function. Previous work with the growing organism may fortify this approach. Firstly, we can now, in some cases, grow cells in good yield in the complete absence of the growth factor by making use of appropriate replacement mixtures; we thus obtain totally instead of partially deficient cells. Secondly, we may obtain hints from replacement studies as to the type of metabolic pathway in which the factor has coenzyme function and even as to the actual step in the sequence of reactions. Thus if C but not B (in Fig. 2) will replace F for growth then F probably functions in the conversion of B to C.

General

The methods of approach to research on biosynthetic pathways which I have tried to piece together in some sort of co-ordinated way arose, of course, from individual experimental observations and their interpretation and confirmation. The realization of their value has come not from theoretical discussion but from frequent use in practice. Some of these observations are milestones in the progress of chemical microbiology; if there had been time it would certainly have been more instructive to follow the development of these ideas rather than to give, as I have had to do, merely a cross-section in very general terms.

In the time which remains a few specific examples will be considered which illustrate a number of these points.

The experimental work on which the preceding discussion is based, the deductions made therefrom, and the difficulties of interpretation which may arise are in the main covered by the following reviews (among others) to whose authors (except himself), and all the workers whose experiments and ideas are included, the lecturer pays grateful tribute:

General nutrition and metabolic function: Lwoff (1944), Knight (1945), Gunsalus (1949), Snell (1951 *a, b*, 1952), Woods (1950 *b*, 1952 *a*).

Inhibitory analogues of metabolites: Roblin (1946), Fildes (1948), Woolley (1952), Shive (1950, 1952).

Biochemical mutants: Beadle (1945), Mitchell (1950), Emerson (1950), Catcheside (1951), Davis (1950, 1952 *a*).

Simultaneous adaptation: Stanier (1950, 1951).

Relationship between purine and histidine

One other type of observation springing from quantitative studies of bacterial nutrition may yield valuable initial information. Suppose that a metabolite such as C (Fig. 2) is required both directly for incorporation into a cell constituent and also as precursor of another such compound D. Then we should expect that the quantitative requirement for C should be less if D is provided preformed in the medium. The work of Snell, who has made so many brilliant contributions to bacterial nutrition, provides an example of this. A strain of *Lactobacillus casei* was found which required guanine for growth, but was able to synthesize histidine. The chemical structures of the two compounds (Fig. 3) suggested to Broquist & Snell (1949) that one might be the precursor of the other, and they found in fact that the requirement for purine was halved by the concurrent presence of histidine (Fig. 3).

* Strain synthesizing histidine under conditions where purine is required for growth.

Fig. 3. The relationship of guanine to histidine. Data interpreted from graphs given by Broquist & Snell (1949).

The function in metabolism of folic acid and its derivatives

The realization of the value of the growth factor replacement technique and the use of inhibitory analogues owes much to the practical help it has given in elucidating the function in the cell of the folic acid group of growth factors. The chemical structures of some of the members of this group are given in Fig. 4; I shall use the term folic acid in general reference to the more complex members of the group, i.e. those containing the pteridine residue, and refer to specific members by the names shown in the figure. Without burdening you with too much chemistry and details of the nutritional requirements of a multiplicity of organisms, the points I wish to make are as follows: (a) all these substances are active with one organism or another as the minimal requirement (within the group) for growth, (b) all of them contain a *p*-aminobenzoic acid residue; the latter is itself active with some organisms

which when given it can complete the synthesis of folic acid, and (c) some form of folic acid, probably more complex in structure than any of those already known, is likely to be the ultimate coenzyme form of the group.

Fig. 4. The structure of some members of the folic acid group. (1) *p*-Aminobenzoic acid; (2) pterioic acid; (3) rhizopterin; (4) pteroylglutamic acid; (5) *N*⁵-formyltetrahydropteroylglutamic acid (probable structure of leucovorin and folinic acid—SF).

It is now generally accepted that the sulphonamides owe their growth-inhibitory property to the fact that they are analogues of *p*-aminobenzoic acid, which is an essential metabolite. *p*-Aminobenzoic acid was in fact first discovered as a substance of biological interest through its ability to overcome sulphonamide inhibition of growth in a competitive manner. In the present sense therefore the sulphonamides may be used as tools for creating an effective deficiency of *p*-aminobenzoic acid, and thus of folic acid, in the systems under test.

The metabolic inter-relationships of *p*-aminobenzoic acid and the various members of the folic acid group are too complex to deal with in detail here. Briefly, experiments with growing cultures and cell suspensions of the kind which I have outlined support the view, first, that all the known functions of *p*-aminobenzoic acid are exerted through its conversion to a coenzyme form

of folic acid, and, secondly, that none of the present known forms of folic acid is the actual coenzyme (or prosthetic group) of the enzymes concerned.

The ability of mixtures of substances (Fig. 5) of quite different chemical type to folic acid to replace *p*-aminobenzoic acid and folic acid for the growth of various organisms, and to overcome inhibition of growth by sulphonamides and analogues of folic acid, gave the first information as to the probable function of folic acid. These substances (certain nucleic acid derivatives, certain amino-acids, vitamin B₁₂) are therefore, by the argument advanced earlier, probable products of the coenzyme-like function of folic acid.

Fig. 5. Scheme showing probable relationship between *p*-aminobenzoic acid, folic acid and the products of their function in metabolism.

These apparent products are many and varied in chemical structure. This suggests that there is some common type of chemical change involving the same coenzyme at some stage of the synthesis of each. As far as has yet been investigated this has proved to be the case, but not all of the specific reactions in the individual synthetic pathways have yet been tracked down and the requirement for folic acid fully established.

In the case of serine and methionine further 'replacement' experiments (see p. 157) with known possible primary intermediates have enabled the particular reaction to be located with some certainty. Thus with serine it is the condensation of glycine with a single-carbon residue, and with methionine the final methylation of homocysteine (Fig. 6). In purine synthesis it was an excretion which gave the first clue. Cultures of *Bact. coli* with restricted available *p*-aminobenzoic acid or folic acid were found to excrete a diamine, first identified as 4-aminoimidazole-5-carboxamide (see Shive, 1950) and very recently shown by gentler methods of isolation to be a riboside derivative of

the amine (Greenberg, 1952). Its possible conversion to purine riboside by incorporation of a single-carbon unit and ring closure is easy to see (Fig. 6).

In our laboratory we* have tried to confirm and extend some of these results at the cell suspension level of investigation. Harvested cells deficient in members of the folic acid group were obtained from growth on suitable replacement mixtures. Synthesis of serine was obtained with suspensions of

Fig. 6. Some reactions in which members of the folic acid group have coenzyme-like function.

various organisms in the presence of glycine, formate and glucose but only if *p*-aminobenzoic acid or folic acid (depending on the organism) were added (Fig. 6). In similar experiments synthesis of methionine from homocysteine by various mutants of *Bact. coli* was found to require both *p*-AB and B₁₂; there was strong evidence that under the test conditions serine is the donor of the required one-carbon unit.

In all three reactions shown in Fig. 6 there is a condensation of a one-carbon unit with the other substrate, and all lead to compounds which are probable products of the function of folic acid. It was first suggested by Shive (1950) and is now a generally accepted hypothesis, that the coenzyme form of folic acid, whatever it may turn out to be chemically, is concerned in the metabolic transfer of single-carbon residues.

It seems likely that further details concerning the coenzyme-like function of folic acid and its mechanism will require suitable systems for investigation at the cell-free enzyme preparation level.

* In collaboration with J. Lascelles, F. Gibson, M. J. Cross, P. M. Meadow and S. Wijesundera.

The experimental work and ideas which have been summarized in a rather general way in this section of the lecture are derived from literally hundreds of papers to which it has unfortunately been impossible to make detailed reference. Full references and detailed discussion will be found in the following reviews and papers: Jukes & Stokstad (1948), Hutchings & Mowat (1948), Winkler & de Haan (1948), Shive (1950, 1951), Woolley (1952), Welch & Nichol (1952), Woods (1952*a*, *b*, 1953).

Biosynthesis of aromatic compounds

The mention of the word 'organic' would, I suppose, bring most frequently to the mind's eye of a microbiologist the picture of a benzene ring. Yet until recently very little was known about the synthesis by living organisms of this simplest of the aromatic structures.

Fig. 7. Stages in the biosynthesis of some aromatic compounds based on the scheme of Davis (1952*a*).

I will take as an example of the value of nutritional studies with biochemical mutants some recent work of Davis on the bacterial synthesis of a number of aromatic compounds (for references see Davis, 1950; 1952*a*, *b*, *c*).

Making use of the ingenious penicillin technique devised simultaneously by himself and by Lederberg & Zinder (1948), Davis isolated a number of mutants of *Bact. coli* with multiple requirements for the five aromatic compounds shown in the lower half of Fig. 7. Various mutants required two, three, four

or all five of this group; the combinations of requirements were always for successive compounds in the order shown (from left to right) and starting with tyrosine. It was unlikely from the frequency of their occurrence, and other evidence, that these strains had arisen as a result of multiple mutations at different loci controlling steps in independent synthetic pathways leading to the five aromatics. It was more likely that the formation of a common precursor (e.g. A in Fig. 7) or precursors of the successive groups of substances had failed.

Search was therefore made among a great variety of aromatic compounds and cyclohexane derivatives for a substance which could promote growth in the absence of some or all of the five compounds. Eventually Stanier, who was also a collector of such compounds for his work by the simultaneous adaptation technique on the oxidation of aromatic substances (see Stanier, 1950), suggested and provided a sample of shikimic acid. This was active with a number (though not all) of the mutants in totally replacing the aromatic requirements; it was probably, therefore, a common precursor of them all.

The role of shikimic acid as an intermediate in aromatic biosynthesis was confirmed by the fact that some of the mutants which do not respond to shikimic acid, and are presumably unable to utilize it, were found to excrete it in considerable quantity. Other mutants of this series were found to excrete different substances; this was detected in the first place by the ability of some mutants to support the growth of others. The identification of these compounds as dehydroshikimic acid and dehydroquinic acid enabled Davis to propose the pathway shown in Fig. 7 for the earlier stages of aromatic synthesis. Great progress has therefore been made, though we are not yet back as far as an open chain precursor.

Further ingenious work (Davis, 1952*b, c*) has provided a satisfactory explanation of why not all the mutants require all the aromatic compounds for growth although the metabolic lesions (as indicated by excretions of the above intermediates) are all in the early common pathway. Strong evidence was obtained that a number of the mutants were not totally deficient in available shikimic acid, and that this substance was used preferentially for the synthesis of *p*-hydroxybenzoic acid, *p*-aminobenzoic acid and so on in decreasing order of priority. Dehydroshikimic acid itself was found to inhibit competitively the utilization of shikimic acid; in a mutant with metabolic lesions both before and after dehydroshikimic acid (so that it was no longer an effective metabolite) increasing concentrations of dehydroshikimic acid relative to shikimic acid induced growth requirements for tyrosine, then phenylalanine plus tyrosine, and so on in the usual combinations. These points illustrate also some of the difficulties which may arise in interpreting nutritional experiments with mutants.

Perhaps I may make a short diversion at a more macrobiological level on the other known biological occurrence of shikimic acid. The sample used initially by Davis had been isolated by H. O. L. Fischer from *Illicium religiosum*. Since this plant has such an enticing name (*ab illiciendo*) I have tried, with the aid of a botanical colleague, to find out something about it. It is a member of

the order Magnoliaceae, and there is a delightful description of it by Hooker in Curtis's *Botanical Magazine* for 1843 (vol. xvi, p. 11)* together with a coloured engraving. After mentioning that this is the sacred aniseed tree of Japan, and that a fine young plant flowered in great profusion in March 1842 in the greenhouse at the Royal Botanic Gardens at Kew, Hooker goes on

By the Japanese this plant is held sacred, they strew wreathes of it and branches over the tombs of their friends, and the priests burn the bark upon the altars of their deities. A singular use is made of the pulverized bark by the public watchmen. Hollow tubes graduated on the outside, are filled with this substance, which is lighted at one extremity, and burns gradually and uniformly: so that when the fire has reached a certain mark, the watchmen strike the hour upon a bell, and thus announce it to the public.

It seems that perhaps after all Bernard Davis was not the first person to ring a bell with shikimic acid!

Pyruvate oxidation factor

My last main example concerns some work which seems to me to illustrate most admirably the interplay of research at the levels of nutrition and metabolism resulting in great progress towards a full solution of the problem.† Furthermore, one phase of these investigations was initiated (see Gunsalus, 1949) with the intention of applying such concepts.

Pyruvate is properly regarded as one of the key intermediates in the metabolism of all types of cell, and thiamine pyrophosphate (aneurin diphosphate) is well known as coenzyme in a number of its metabolic transformations. One such reaction brought about by bacterial as well as animal cells is its oxidative decarboxylation to acetate and CO_2 (Fig. 8). Gunsalus, O'Kane and their colleagues found that cells of a strain (10 C 1) of *Strep. faecalis* harvested from a complex medium had high activity in this reaction. But cells harvested from a chemically defined medium, which supported good growth, were almost inactive. The activity of the latter suspensions was restored by the addition of yeast extract, but not by any of the known vitamins. It appeared, therefore, that some new and unknown coenzyme-like factor was concerned in the oxidation of pyruvate by this organism, and it was named pyruvate oxidation factor (P.O.F.) (O'Kane & Gunsalus, 1948; O'Kane, 1950; Gunsalus, Dolin & Struglia, 1952; Gunsalus, Struglia & O'Kane, 1952). Highly active concentrates from yeast extract were obtained.

Meanwhile work had been in progress in other laboratories on two unidentified growth factors for micro-organisms (Fig. 8). First, Snell and his colleagues had found that acetate stimulated the early growth of several lactobacilli and could be replaced by minute amounts of yeast extract (Guirard, Snell & Williams, 1946*a, b*). Secondly, a factor (later called protogen) present in liver and required for the growth of the ciliate *Tetrahymena geleii* W had

* I am much indebted to Dr J. H. Burnett of the Department of Botany, Oxford, for tracing this reference.

† Since much of this work is rather recent, and there are no comprehensive reviews, references to the original literature will be given in this section.

been discovered by Kidder and his group (see Kidder & Dewey, 1949, 1951) and its concentration and isolation pursued by the Lederle group of workers (Stokstad, Hoffmann, Regan, Fordham & Jukes, 1949). It became clear that these two factors had similar properties both to one another and to pyruvate oxidation factor; they were found to be largely interchangeable in their biological effects (Snell & Broquist, 1949; Reed, De Busk, Gunsalus & Hornberger, 1951).

Fig. 8. Scheme illustrating the relationship and biological activities of pyruvate oxidation factor and related growth factors.

While these factors were being purified several other organisms (listed in Fig. 8) were found to require the new vitamin for growth (Stokstad, Hoffmann & Belt, 1950; Lytle & O'Kane, 1951; Kline, Pine, Gunsalus & Barker, 1952).

Two groups of workers (Gunsalus, Reed and their colleagues and the Lederle team) each succeeded in isolating crystalline material from natural sources. They were in pursuit respectively of pyruvate oxidation factor and protogen, but each used a variety of the biological effects (Fig. 8) in their test systems. They called their products α -lipoic acid and protogen B (see Reed, Gunsalus, Schnackenberg, Soper, Boaz, Kern & Parke, 1953; Patterson, Brockman, Day, Pierce, Macchi, Hoffmann, Fong, Stokstad & Jukes, 1951). Examination of the chemical properties, and finally the synthesis of active material, showed that the structure was that of a dimercapto-*n*-octanoic acid (Fig. 8), but the exact position of the sulphur atoms is not yet established. The groups of active synthetic compounds are referred to as synthetic lipoic acids and thioctic acids respectively (Hornberger, Heitmiller, Gunsalus, Schnackenberg & Reed,

1953; Bullock, Brockman, Patterson, Pierce & Stokstad, 1952). Conjugated or bound forms exist in nature from which the new factor is released by acidic or enzyme hydrolysis (Reed, De Busk, Johnston & Getzendaner, 1951; Gunsalus, Struglia & O'Kane, 1952).

Coenzyme form. It would be in accord with usual experience in this sort of work if the substance first recognized as a growth factor were a less elaborate molecule than the ultimate coenzyme form (as, for example, with pyridoxin and *p*-aminobenzoic acid). Furthermore, an organism might well be found which was unable to complete the synthesis of the more complex form (possibly one of the conjugates mentioned above) and required it as a growth factor.

Fig. 9. The relation of lipothiamide and its pyrophosphate to α -lipoic acid and thiamine. Based on the work of Reed & De Busk (1952b).

Reed & De Busk (1952*a, b*) made a deliberate search for such an organism among mutants of *Bact. coli* responding to yeast extract. They found one which would not grow with free α -lipoic acid, but which did so when supplied with one of the complex forms with activity as acetate-replacing factor for *Strep. lactis*. They had therefore a very useful tool for further investigations of the nature of the higher form.

The next step was to identify the other part or parts of the active conjugate molecule. Thiamine (Fig. 9) was a possibility in view of its known role in pyruvate metabolism. Again at the level of growing cultures Reed & De Busk (1952*b*) found that the addition of acetate to the medium for *Strep. lactis* not only abolished the need for α -lipoic acid (as expected) but also that for thiamine. It was likely therefore that acetate was, under the test conditions, a product of the joint function of the two growth factors. They next went to the cell suspension level and incubated harvested cells of the same organism with α -lipoic acid and thiamine; the reaction products supported the growth of the mutant strain of *Bact. coli* but only if both factors had been present

during the incubation. *Streptococcus lactis* could therefore synthesize the form required by the mutant from α -lipoic acid and thiamine. Finally, Reed & De Busk obtained material with similar activity by heating the two substances together *in vacuo* at 130° and by other methods. Chemical evidence showed an amide link between the two molecules and the structure indicated in Fig. 9 was proposed; the compound was called lipothiamide.

The function of lipothiamide in pyruvate metabolism of *Bact. coli* was shown by the use of cell suspensions of the mutant deficient in this factor. These were obtained from cultures on a medium containing casein hydrolysate (which apparently supplies all the products of the coenzyme function), but no lipothiamide. The deficient cells had negligible pyruvate oxidase activity unless lipothiamide were added; the component vitamins, either alone or in admixture, were inactive.

Extending this work finally to the level of cell-free enzyme preparations, Reed & De Busk (1952c, 1953) have recently reported that the extracted apoenzyme (from deficient cells) could not be reactivated even by lipothiamide, but required the pyrophosphate ester (Fig. 9) which may therefore be the actual coenzyme.

In connexion with this work it is interesting to note that Peters, Stocken & Thompson (1945) had suspected some time earlier that a dithiol compound was a component of the pyruvate oxidase system of animal tissues from their work on the inhibition of this enzyme by trivalent arsenicals (such as lewisite), and the removal of such inhibition by the dithiol compound British anti-lewisite (BAL).

*Some further considerations concerning metabolic function
of growth factors*

Even when a function has been discovered for a growth factor in some metabolic event within the cell it still does not prove that it is this reaction and function which is of critical importance for growth. This point has been well stressed recently by Snell (1952) who has shown actually in experiments with growing cultures that several of the known reactions in amino-acid metabolism in which vitamin B₆ derivatives have coenzyme function are indeed important for growth.

It is also necessary to consider whether a B-group vitamin may not have other effects which are far from catalytic. Dr Moulder and I were recently surprised to find that the growth of a strain of yeast (*Saccharomyces cerevisiae* Y 47), with which we happened to be working, was potently inhibited by a mixture of adenine and nicotinic acid (Moulder & Woods, 1953). Both these compounds are certainly essential metabolites within the usual meaning of the word. Adenine is indeed required for the growth of this organism in the absence of *p*-aminobenzoic acid, while nicotinic acid (or a derivative) is demonstrably synthesized. The concentrations required for inhibition (Fig. 10) are only of the order required for growth by organisms which cannot synthesize them.

These results recalled to us a similar and even more striking case discovered

by Rabinowitz & Snell (1951) with another species of yeast (*Saccharomyces carlsbergensis* 4228). Thiamine, which is not required as an exogenous growth factor, actually inhibited growth at concentrations (Fig. 10) which are certainly as low, if not lower, than those required by exacting organisms.

The inhibitions were in both cases overcome by traces of vitamin B₆ (Fig. 10); the latter is not normally required as a growth factor by either organism. So that the presence of the other metabolites had induced a re-

	Growth inhibited 50% by	Full growth restored by
<i>Sacch. carlsbergensis</i> 4228	10 ⁻¹⁰ M-thiamine	10 ⁻¹⁰ M-pyridoxal
<i>Sacch. cerevisiae</i> Y47	10 ⁻⁶ M-adenine and 10 ⁻⁶ M-nicotinic acid	10 ⁻⁹ M-pyridoxin

Neither yeast requires pyridoxal or pyridoxin for full growth on its basal medium.

Fig. 10. Growth factors as inhibitors of the growth of yeast. Data obtained from Rabinowitz & Snell (1951) and Moulder & Woods (1953).

quirement for vitamin B₆. This is reminiscent of the inhibitory inter-relationships between amino-acids first observed by Gladstone (1939) in studies of the nutrition of the anthrax bacillus.

There is no explanation yet to hand for these inhibitory effects of vitamin B-group substances on the growth of yeast, but it certainly seems that substances known as growth factors may have, at least in growing cultures, quite different effects to those discussed earlier in the lecture. Perhaps such effects may play a part in the co-ordinated control of interwoven metabolic pathways.

A recapitulation

Lest we become a little too complacent after reviewing some of the achievements of biochemical microbiology, let us examine again some of the methods we have used to attain our ends.

To begin with there is certainly no doubt that the microbes worked with by biochemical microbiologists must be considered a privileged class! To study their growth, or to obtain our cell suspensions, we normally put them into a rich environment, which, whether chemically defined or not, contains an abundance of everything we think they may want. Here they live a life of ease and luxury for perhaps twenty cell generations, during which time mutants may be selected (or the cells adapt enzymically) so that they are specially suited to this comfortable life. Have we any right to assume that by studying them we can get a true picture of the life of their working class cousins in, let us say, the soil? It seems that long ago Winogradsky had similar misgivings in the realms of purer microbiology (see Stanier, 1953; Winogradsky, 1949).

Perhaps I may quote one example from our own recent experience. My colleague Wijesundera and I were extending to other strains of *Bact. coli* some

work of this Unit on the synthesis of methionine from homocysteine by cell suspensions of this organism. A strain requiring *p*-aminobenzoic acid was grown in a replacement mixture (containing methionine) in order to obtain cells deficient in the vitamin. One strain, tested after harvesting from such a medium, synthesized almost no methionine, whereas when it was grown in the absence of methionine (presence of *p*-aminobenzoic acid) it synthesized well. The effect was found to be due to methionine itself and was proportional to the methionine concentration during growth (Wijesundera & Woods, 1953). A single overnight sub-cultivation in the presence of methionine had led to an almost complete loss of ability to synthesize this amino-acid. A similar effect, of a considerable though variable extent, was found with a number of other strains of the organism. Cohn, Cohen & Monod (1953), who were studying methionine synthesis from a rather different point of view, have concurrently observed the same phenomenon.

In the preface to the first edition of her book *Bacterial Metabolism*, Marjory Stephenson (1980) surveyed the general position and methods of investigation in our subject in 1930. She then wrote

We are indeed in much the same position as an observer trying to gain an idea of the life of a household by a careful scrutiny of the persons or material arriving at or leaving the house: we keep accurate record of the foods and commodities left at the door and patiently examine the contents of the dustbin and endeavour to deduce from such data the events occurring within the closed doors.

What is the position now after a further twenty-three years? I do not think that we have yet succeeded in opening the door and paying a friendly visit. But we have certainly devised some ingenious methods of what I can only call 'peep-tomming'—or should it be 'tom-peeping'? These methods are, however, strangely reminiscent of the atomic age that grew up parallel with them. We subject the inhabitants of the house to noxious radiations and mustard gas and cause them to mutate. We shatter and disintegrate the house by violent physical means, and we study the debris on the lawn and the feeble and unco-ordinated flutterings of such signs of life as we find. We blockade the house and starve the inhabitants by preventing the delivery of essential foodstuffs. Worse still, we subtly fashion loaves of clay and bribe the baker's boy to deliver them instead of bread! For this ultimate beastliness, 'this further innovation in cell torture' as Marjory Stephenson (1946) once called it in referring to some work of my other chief Sir Paul Fildes (1941), I suppose that I must myself bear a large measure of responsibility.

Perhaps it is, after all, not without reason that our well-meant efforts are sometimes referred to as 'microbating' by our more biological colleagues.

By all this my real intention is to renew the plea that we should check our results, whenever possible, at the more biological end of our spectrum of methods of investigation.

If I have concluded this lecture on a lighter note, it is only because my long association with 'M.S.' (as she was always affectionately known to us) taught me clearly that it was equally part of her philosophy that in chemical microbiology there should be some measure of gaiety as well as good science.

This lecture is dedicated, in all humility, to the memory of the late Dr Marjory Stephenson, F.R.S.

I am deeply conscious of the debt that I owe, not only to her, but also to Sir Paul Fildes, F.R.S. and Sir Rudolph Peters, F.R.S. for all that they have made known to me of the deeper meaning of microbiology and biochemistry. To many colleagues in this and other lands, and especially to Dr D. W. Woolley, I am indebted for fruitful discussion of some of the subjects considered in this lecture. Finally, I acknowledge with gratitude the willing help given by my colleagues in the Microbiology Unit of this Department both in assembling the material and debating some of the points which arose.

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