VITAMIN-B-GROUP SUBSTANCES,
ESPECIALLY FOLIC ACID AND B_{12},
IN THE METABOLISM OF MICRO-ORGANISMS

D. D. WOODS, M.A., Ph.D., F.R.S.

Reader in Microbiology,
Oxford University, England

The common requirement of all types of living cells for vitamin-B-group substances for their normal metabolism and growth must reflect a fundamental similarity in basic cell-processes. The function of these substances, as far as is known at present, appears to be as components of catalytic systems. Many of them have turned out to be a part of the structure of co-enzymes or prosthetic groups of important enzyme systems; in the case of factors of unknown function, it is now justifiable to adopt such a possibility, at least as a working hypothesis. Naturally, such deductions have been based on evidence from work with animal as well as bacterial cells, but a number of the initial key observations have come from studies of bacterial nutrition and metabolism. This is particularly the case with those B-vitamins which are ultimately concerned with the synthetic rather than the catabolic processes of the cell, and it is the main purpose of this paper to state and illustrate the methods which have been used in making such advances. Some of these methods are applicable only to microorganisms because of their rapid rate of multiplication and consequent synthesis of new cell-material. The simplicity of the experimental criterion of vitamin deficiency, i.e., failure to multiply, has also been of considerable value.

GENERAL METHODS OF INVESTIGATION

The methods of approach which have been fruitful in studies of growth-factor function with bacteria have been derived from work with both growing cultures and with cell suspensions.

A. Studies of Bacterial Nutrition

(1) Replacement of requirement for a B-vitamin by a substance of a different chemical type

This type of approach is best made clear by consideration of a hypothetical series of reactions (A→B→C) by which is synthesized the substance C which is essential for the normal growth of the organism (see fig. 1). Let
us suppose that the growth-factor (F) is a precursor of the co-enzyme (Co) or prosthetic group of the enzyme (E) concerned in the transformation of B to C. When F (which the organism cannot synthesize) is absent from the medium, then growth fails. Growth, however, should occur if a pre-formed source of C is supplied, even if F is absent. When, therefore, it is found that a growth-factor can be effectively replaced by a substance of a quite different chemical type, there is suggestive evidence that the growth-factor is concerned with the formation of that substance from its precursors. If F is concerned (as Co) in the transformation of Y to Z, as well as of B to C, then the provision of C may reduce, though not abolish, the requirement for F. In such a case, only a mixture of all such substances (e.g., C, Z) would totally replace F. This method of approach is limited to organisms which require the vitamin for growth.

(2) Inhibition of the utilization of a B-vitamin by an analogue

Growth of a micro-organism is often prevented by the presence of a chemical analogue (see F' in fig. 1) of a growth-factor. There is much evidence that this is usually due to competition between F and F' for the enzyme concerned in the conversion of F to Co. In any event, the result is effectively the same as in A (1) above; the conversion of B to C is no longer possible and growth does not occur. Inhibition may usually be overcome in a competitive way by the addition of F. Theoretically, and often in practice, inhibition may also be overcome by the addition of either: (a) Co, or a simpler intermediate between F and Co of related structure to F', or (b) by the products (C, Z) of the reactions dependent on Co. In either case, inhibition of growth by F' would be overcome in a non-competitive manner, i.e., the organism would be insensitive to any reasonable concentration of the inhibitor. This follows since the products (direct or indirect) of the utilization of F have already been provided preformed. This method of approach evolved from the discovery that the sulfonamides inhibit bacterial growth by preventing the utilization of the structurally analogous growth-factor, p-aminobenzoic acid (PAB); it has also proved useful with other growth-factors. In principle, it is essentially similar to the first method; an effective lack of the vitamin is induced, in some cases even though the organism is normally able to synthesize the vitamin. Discovery of substances of a different chemical type from F, which permit the organism to grow again, suggests an ultimate function of F in the synthesis of these substances.

B. Metabolism of Cells Deficient in the Vitamin

This method of approach was first used by Peters & Thompson in their work on the function of vitamin B1 (thiamine) in the pigeon. Bacterial cells, partially or wholly deficient in a given vitamin, may readily be obtained by growth in a medium containing only suboptimal amounts of the factor, or one in which the factor is replaced as described in A (1) above. The
metabolism of such deficient cells is then compared in detail with cells harvested from media rich in the vitamin. Partial or total failure of the deficient cells to carry out certain reactions may indicate a function of the vitamin in those reactions, though it must be realized that the function may be indirect. Failure of one reaction may lead to failure of other reactions which depend on the product of the first reaction, either as a primary substrate or as a co-enzyme. The specificity of the effect is made clearer if the addition of the vitamin to the harvested deficient cells restores their ability to carry out the reaction; cell suspensions may not, however, be able to convert the free vitamin to the co-enzyme form, especially if the latter is much more complex in chemical structure.

**FIG. 1. SCHEME ILLUSTRATING HOW INFORMATION CONCERNING METABOLIC FUNCTION OF GROWTH-FACTORS MAY BE OBTAINED FROM NUTRITIONAL STUDIES WITH MICRO-ORGANISMS**

\[
\begin{align*}
  & F \\
  & F' \\
  & Co + Enz \\
  & A \rightarrow B \rightarrow C \\
  & B_i \\
  & Y \rightarrow Z \\
\end{align*}
\]

- \(A, Y\) = initial substances
- \(B\) = intermediate
- \(C, Z\) = substances essential for normal growth of organism
- \(F\) = growth-factor
- \(Co\) = co-enzyme
- \(F'\) = chemical analogue of growth-factor

**C. Metabolism of the Vitamin**

The substance active as a growth-factor or vitamin has often proved not to be the form in which it has a final function in cell metabolism. It is often, for example, a simpler molecule which forms only a part of the structure of the co-enzyme (see fig. 1). Study of the metabolism of the factor itself, and the linkage of such metabolism to other cell-processes, may throw light on the question as to whether the factor must first be converted to a more complex form. Deficient cells (see section B) which are not activated by the factor itself may be so activated if given preliminary treatment with the factor, together with an energy source and possibly other substrates.
D. Replacement of Co-enzymes

Final proof of the ultimate catalytic function of B-group vitamins can be obtained only by the isolation of the enzyme system implicated in a cell-free condition, followed by its purification, dissociation, and reactivation by the suspected co-enzyme form of the vitamin. Knowledge of the structure of the co-enzyme is usually also required before its actual mechanism of function can be elucidated.

Conclusions

It is clear that any of the above methods of approach may give misleading results in the sense that the function indicated may be indirect. Results are therefore to be regarded as a guide for future work rather than as a proof; evidence obtained by one method should always be checked by as many other methods as possible.

It is only possible here to illustrate the results that may be obtained by such investigations by detailed reference to work on the function of one group of bacterial growth-factors (folic acid and related compounds). Vitamin B₁₂ will also be considered to some extent, however, since it appears to be concerned in the synthesis of several of the compounds implicated for folic acid.

It is also necessary to restrict the discussion mainly to work with microorganisms, mentioning only briefly a few relevant results with animal tissues.

It is clear that the realization of the value of these lines of approach, and indeed their elaboration, has evolved only through their application to specific problems. Studies of the function of PAB and folic acid have contributed a good deal to the development of the approaches listed under section A.

METABOLIC FUNCTION OF FOLIC-ACID GROUP OF FACTORS

The work to be reviewed here covers several hundred papers and it will be necessary to refer mainly to those reviews from which detailed references may be obtained. The term "folic acid" will be used in general reference to the group of factors, both natural and synthetic, as a whole; pteroylglutamate (PtG) will be used for the synthetic material active for Lactobacillus casei but not for Leuconostoc citrovorum. The natural factor for Ln. citrovorum will be called "citrovorum factor" (CF) and the corresponding synthetic material folinic-acid-SF.
Relation of p-Aminobenzoic Acid to Folic Acid

The biological importance of p-aminobenzoic acid (PAB) was first realized not through any direct effect on bacterial growth, but because of its ability to overcome the inhibition of such growth by the sulfonamide drugs. Detailed examination of this property led to the hypothesis that PAB is an essential metabolite for micro-organisms, and that its utilization by the cell is competitively inhibited by the sulfonamide drugs by virtue of their similarity in chemical structure. This hypothesis has now received ample experimental support—in particular, from the discovery that PAB is an essential growth-factor for a wide variety of micro-organisms—and is now generally accepted (Woods 59). A knowledge of the function of PAB in the cell (i.e., of the products of its utilization whose formation is inhibited by sulfonamides) was therefore required for further understanding of the action of these drugs.

There is now very strong evidence that the main, if not the only, function of PAB is for the synthesis of folic acid and related substances which, in turn, are essential metabolites for bacteria. All known members of this group contain a PAB residue in the molecule (see fig. 2). Pteroylglutamic acid was synthesized in 1946 by the Lederle and American Cyanamid Company group of workers, and was shown to have the full biological activity of the growth-factor for Lactobacillus casei present in liver. Soon after, an unknown growth-factor for Streptococcus faecalis R (rhizopterin) was found by the Merck group of workers to be replaced by the synthetic N\textsuperscript{10}-formylpterioic acid. More recently, it has been found that the growth-factor for Ln. citrovorum is a formyl derivative of reduced pteroylglutamic acid; a synthetic product with the structure shown in fig. 2 (N\textsuperscript{5}-formyl-tetrahydropteroylglutamic acid) has high biological activity. Detailed references to this work will be found in Jukes & Stokstad; 20 Hutchings & Mowat; 18 Broquist, Stokstad & Jukes; 3 and Pohland, Flynn, Jones & Shive. 36

If PAB is required only for the synthesis of folic acid it would be expected:

(a) that organisms requiring folic acid for growth would not be inhibited by sulfonamides,

(b) that folic acid would replace PAB for growth of organisms requiring the latter factor, and

(c) that, in the presence of folic acid, all organisms, whether requiring PAB or not, would be insensitive to sulfonamides.

Expectation (a) has been fulfilled. However, in experiments using PtG as source of folic acid, expectations (b) and (c) have been fulfilled with some organisms, but not with others (Woods 59). Similar results have been obtained with synthetic folinic acid except with one organism (Woods,
unpublished material). *Leuconostoc mesenteroides*, which requires PAB for growth, responds to folinic-acid-SF though not to PtG; furthermore, sulfonamide inhibition is overcome in a non-competitive manner by folinic acid (Lascelles, Cross & Woods 24).

The inactivity of folic acid with a number of organisms remains to be explained. Representative organisms of this type (*Staphylococcus aureus*, PAB-requiring mutant of *Escherichia coli*) nevertheless synthesize a folic acid during growth and in cell suspensions, as does also *Streptobacterium plantarum*, an organism for which PtG is active in replacing PAB for growth. In all these cases the synthesis is dependent on PAB and is inhibited by sulfonamides (Nimmo-Smith, Lascelles & Woods; 32 Lascelles & Woods 26).

**FIG. 2. STRUCTURE OF VARIOUS FOLIC-ACID DERIVATIVES**

1. *p*-Aminobenzoic acid
2. Pteroyl acid
3. Rhizopterin
4. Pteroylglutamic acid
5. Probable structure of folinic-acid-SF (N-formyl-tetrahydropteroylglutamic acid)
It will also be seen later that both PAB and folic acid have, as far as is known, the same ultimate function in cell metabolism. A reasonable explanation of these complexities which fits the facts known at present is shown diagrammatically below:

Neither PtG nor folinic acid may be a direct intermediate in the conversion of PAB to the ultimate co-enzyme form of folic acid, which may well be more complex in structure than any known form. PtG may be readily converted to a true intermediate X by some organisms, but not by others. Folinic acid may be in similar relationship to the higher intermediate Y; this would imply that *L. citrovorum* and *L. mesenteroides*, but not other organisms, are able to convert folinic acid to Y and thus on to F.

There remains, also, the possibility that PAB has a separate function in cell metabolism not exercised through the intermediate formation of a folic acid. If this is so, it would appear that such a function is required only for the growth of some organisms.

The complexities in the precise metabolic relationship of PAB and folic acid do not impede a close study of their cellular function; such studies may indeed aid in clarifying the matter.

**Replacement of p-Aminobenzoic Acid and Folic Acid by Amino-acids and Nucleic-Acid Derivatives**

Much information concerning the function of the group of factors as a whole has come from work with growing cultures by the approaches described in sections A(1) and A(2). The sulfonamides have been used as
specific inhibitors of the utilization of PAB, and various analogues of folic acid (such as aminopterin) as inhibitors of that factor. Most of this work has been reviewed in detail by Shive and Woods. Briefly, certain mixtures of amino-acids and nucleic-acid derivatives have been found to replace PAB and folic acid, either wholly or in part, for the growth of organisms requiring those factors. Similar mixtures overcome the inhibitory action of sulfonamides and analogues of folic acid, usually in a non-competitive manner. These observations therefore strongly suggest that the PAB/folic-acid group is concerned in the biogenesis of such compounds.

The substances implicated are:

(a) thymine or, more probably, its desoxyriboside, thymidine;
(b) purines—the precise purine or purines vary somewhat with the organism tested;
(c) amino-acids, particularly methionine and serine; with various organisms, lysine, histidine, threonine, leucine, and other amino-acids also have considerable activity;
(d) vitamin B₁₂, which contains, inter alia, a benziminazole derivative (an analogue of purine) in the molecule.

Three examples will serve to illustrate these phenomena.

(1) Replacement of the growth-factor function of PAB

Lampen, Jones & Roepke found that a mutant strain of Esch. coli requiring PAB could grow in the complete absence of this substance if the medium contained thymine, a purine (four purines tested were equally effective), and a mixture of amino-acids. Of the latter, methionine was essential, while serine, threonine, lysine, histidine, and tyrosine each greatly increased the rate of growth in the presence of methionine. Growing under these conditions the organism was almost insensitive to sulfonamides.

(2) Replacement of the antisulfonamide function of PAB

With normal strains of Esch. coli, Winkler & de Haan found that the amount of PAB required to overcome inhibition by a given concentration of sulfanilamide was progressively diminished as methionine, xanthine, and serine (in that order) were added to the simple basal medium; the further addition of thymine eliminated the requirement for PAB, i.e., the organism was no longer sensitive to the drug.

(3) Replacement of the growth-factor function of folic acid

Broquist and Snell studied the folic-acid requirement of Strept. faecalis R in a medium containing purines and all the amino-acids except serine. The addition of serine reduced the amount of PtG necessary for growth by a factor of 10, while the further addition of thymine abolished the requirement.
Evidence of this kind suggests a function of PAB/folic-acid in the overall synthesis of the substances in question, but gives no hint as to which step in the synthesis is catalysed by the factors, nor any indication of the mechanism of their catalytic function. A report of the progress made in these respects for particular substances follows.

**Function in the Synthesis of Nucleic-Acid Derivatives**

*General*

The possible role of folic acid at some stage in the synthesis of nucleic acid has received general confirmation from observations of the nucleic-acid content of cells grown in media deficient in this factor. Schopfer found that cells of a strain of Saccharomyces contained less ribonucleic acid when grown in the presence of sulfathiazole, and therefore in a medium effectively deficient in PAB. Growth of *Lb. casei* in a medium deficient in folic acid produced cells with a smaller content of desoxyribonucleic acid than normal cells; the ribonucleic-acid content was not affected (Prusoff, Teply & King).

**Accumulation of 4-aminoimidazole-5-carboxamide**

Suggestive evidence as to the stage at which folic acid is required for purine synthesis has come from the accumulation in the growth medium of a possible precursor of purines when there is an effective deficiency of PAB or folic acid. A diamine which accumulated in cultures of *Esch. coli*, partially inhibited with sulfonamide, was identified as 4-aminoimidazole-5-carboxamide by Shive et al. who suggested that PAB, or some compound derived from it, e.g., folic acid, is a co-enzyme for its conversion to purine by the addition of a single-carbon unit and ring-closure (see fig. 3). Woolley & Pringle have found a similar accumulation of the carboxamide when growth is partially inhibited by 4-aminopteroylglutamic acid (a folic-acid

---

*a* However, Chang, Silverman & Keresztesy have recently reported that cells of *Ln. citrovorum* deficient in CF have a higher concentration of ribonucleic and desoxyribonucleic acid than normal cells.
analogue). The position of the carboxamide as a purine precursor is strengthened by the recent work of Gots, who found it present in cultures of a purine-requiring mutant of *Esch. coli*. Furthermore, glycine (a probable precursor of purines in animal tissues) has been shown to increase greatly the production of the carboxamide by cultures of *Esch. coli* (Ravel, Eakin & Shive 38).

The carboxamide, at rather high concentrations, is able to replace purine for growth of some organisms but not others (Shive; Gots 14). R. H. Nimmo-Smith (unpublished observations made in this laboratory) has found it active in this sense in 4 out of 10 organisms tested, though 20 to 200 times the effective concentration of purine is required. He also found that the corresponding carboxamidine, which has the same chemical relationship to adenine that the carboxamide has to hypoxanthine, was effective with all ten organisms, though, again, concentrations of the same magnitude as the carboxamide were necessary. The high concentrations required may indicate that the cell is less permeable to these substances than to purine; it is also possible that some derivative, perhaps indeed the riboside or desoxyriboside, is the true intermediate, and that the actual product after ring-closure is the nucleoside rather than the free purine. In the case of the pyrimidine, thymine, there is indeed strong evidence that the desoxyriboside is the product of the reaction which folic acid catalyses.

**Thymidine as product of the utilization of folic acid**

Two examples will serve to illustrate the type of evidence (again, replacement studies with growing cultures) which supports this view. Sauberlich & Baumann 43 found that thymidine, rather than thymine, was active in replacing CF for the growth of *Ln. citrovorum* in a medium already containing amino-acids and purines. With a strain of *Ln. mesenteroides*, inhibition of growth by methylpteroylglutamic acid was overcome by thymidine (and PtG), but not by thymine (Shive, Eakin, Harding, Ravel & Sutherland. With another strain of the same organism, June Lascelles (unpublished material) found that thymidine, but not thymine, eliminated the need for PAB in an amino-acid medium containing purines.

**Conclusions.** The evidence so far obtained is in accord with Shive’s suggestion of a co-enzyme function of folic acid in the addition of a single-carbon residue in the final step in the synthesis of the purine nucleus, though the precursor may be condensed with a sugar residue before such incorporation. There is no similar evidence so far for an analogous precursor in pyrimidine synthesis, though the work of Mitchell & Houlanah on a series of *Neurospora* mutants requiring uracil suggests this may be so; aminofumaric acid diamide replaced uracil with some mutants.
Vitamin $B_{12}$ in the synthesis of nucleosides

Nutritional experiments with lactobacilli which need vitamin $B_{12}$ for growth suggest that this factor is concerned in the synthesis of purine and pyrimidine deoxyriboside structures, though in a different way from folic acid. Briefly, the vitamin-$B_{12}$ requirement of such organisms as *Lb. leichmannii*, *Lb. lactis* Dorner, and *Lb. acidophilus* (some strains) can be met by thymidine or, in most cases, by various purine deoxyribosides. The latter property is in sharp contrast to that found (with the appropriate organisms) for the replacement of folic and folinic acids, where thymidine or thymine is always required in addition to purines, and where purine deoxyribosides alone are inactive. Details of some of this work and references to other papers are given by Kitay, MacNutt & Snell,\textsuperscript{21} and Jukes, Broquist & Stokstad.\textsuperscript{19} Of great interest in connexion with this problem is the recent demonstration by MacNutt\textsuperscript{27} of the existence in *Lb. helveticus* of enzymes catalysing the reversible transfer of the deoxyribosyl group from pyrimidines to purines.\textsuperscript{b}

The separate functions of folic acid and $B_{12}$ in metabolism are emphasized by the fact that they are not interchangeable for the growth of any organism requiring either one or the other. Indeed, *Lb. leichmannii* requires both folic acid and vitamin $B_{12}$. In the absence of $B_{12}$, growth can be obtained on addition of either thymidine or a purine deoxyriboside, but folic acid can be replaced, with limited growth, only by thymidine. With both factors missing, limited growth was obtained with thymidine alone. Since it was also found that *Ln. citrovorum* (requiring CF, replaced only by thymidine) could synthesize $B_{12}$, and *Lb. leichmannii* (requiring $B_{12}$, replaced by any deoxyriboside) could synthesize CF from folic acid, Jukes et al.\textsuperscript{19} suggested that $B_{12}$ has a function in the synthesis of deoxyribosides of purines and cytosine, while CF is required specifically for the origin (reversible) of thymidine from the other deoxyribosides.

It is unlikely that the respective functions and interrelationships of folic acid and $B_{12}$ in nucleic-acid synthesis will be further clarified until simpler systems than growing cultures (e.g., cell suspensions or enzyme preparations) can be used for more direct experiments. The situation is further complicated by the possibility that folic acid is, itself, also concerned at some stage in $B_{12}$ synthesis (Shive; \textsuperscript{46}Davis\textsuperscript{9}); it is unlikely, however, that such a function could explain the results just described. Meanwhile, the nutritional approach has provided a valuable starting-point for future work.

Function in the Synthesis of Amino-acids

During recent years, part of the work of this laboratory has been concentrated upon an attempt to analyse the function of PAB and folic acid in

\textsuperscript{b} This work has recently been published in full by MacNutt\textsuperscript{14} and extended to two other lactobacilli giving a growth response with deoxyribosides.
amino-acid synthesis, using whenever possible simpler systems than whole growing cultures. Of the amino-acids implicated by nutritional studies as probable products of the catalytic function of PAB and folic acid, methionine and serine offered the best hope for further investigation since there was considerable knowledge regarding intermediates in their biosynthesis. There was thus more hope of implicating the growth factors in specific cell reactions.

**Synthesis of serine**

*Glycine as a probable precursor.* Induced mutant strains of *Neurospora* and *Esch. coli* are known which require either serine or glycine for growth (Tatum; Roepke, Libby & Small). It is not clear from these experiments which amino-acid is the ultimate growth-factor and which serves as precursor of the other. When *Torulopsis utilis* was exposed for a short period to glycine (labelled with isotopic C in the -COOH group) as the sole source of carbon, Ehrensvärd et al. found the bulk of the radioactive carbon in the -COOH group of the serine fraction of the hydrolysed cell protein.

Experiments with animal tissues using isotope-labelled substrates show that serine can arise from the condensation of glycine with a single-carbon compound such as formate:

\[
\text{H.CO}OH + \text{CH}_2\text{NH}_2\text{COOH} \rightarrow \text{CH}_2\text{OH.CHNH}_2\text{COOH}
\]

Thus, labelled carboxyl carbon from glycine was found in the carboxyl group of serine, while labelled carbon of formate was found in the β-carbon of serine. Other substances besides formate, e.g., formaldehyde, acetone, choline, and glycine itself, can exchange a carbon atom with the β-carbon of serine, and therefore act as donors of a single-carbon residue in the condensation reaction with glycine (Sakami; Siegel & Lafaye). In none of these experiments was an actual increase in the amount of serine present in the test system demonstrated; if the reactions are reversible (as seems to be the case), isotope exchange could occur without increment of serine.

While the experiments described below were in progress, Plaut, Betheil & Lardy found that the incorporation of \(^{14}\text{C}\) from labelled formate into the β-carbon of serine was markedly less in folic-acid-deficient rats than in normal animals. This suggested that folic acid was concerned either in the activation of formate or in its condensation with a two-carbon compound, presumably glycine, to form serine.

*Streptococcus faecalis* R was chosen as the test organism for the present work because nutritional experiments of the type described earlier in this

---

\(^{c}\) I am grateful to my colleagues Dr. June Lascelles, Mr. M. J. Cross, and Mr. F.W. Gibson for permission to quote from their unpublished results.
paper (see page 36) had shown that it should be possible to obtain harvested cells completely devoid of folic-acid and vitamin-B₉ derivatives, simply by growth in the presence of those substances in whose synthesis the two factors appear to be concerned.

_Growth experiments with_ Streptococcus faecalis _R_. This organism requires serine for growth. Higher concentrations of glycine (five times that of serine) will replace serine, but only if both pyridoxal and PtG are provided. Furthermore, the need for PtG is eliminated by the joint presence of thymine and serine, but not by thymine and glycine (Lascelles & Woods, unpublished material). These experiments confirm that, with this organism too, glycine (or a substance derived from it) is the immediate precursor of serine, and provide strong evidence that both folic acid and pyridoxal are required for the conversion of glycine to serine.

_Synthesis by cell suspensions of_ Streptococcus faecalis _R_. The organism was grown on a defined medium containing purines and a complete amino-acid mixture, and supplemented with DL-alanine and thymine. Under these conditions neither PtG nor pyridoxal is required for good (though not optimal) growth, and the harvested cells are completely deficient in both factors. Traces of PtG were normally added to the medium to promote more-rapid growth; the cells were still devoid of folic acid.

Washed suspensions of such cells synthesized serine in a system containing buffer, glucose, glycine, formate, PtG, and pyridoxal (Lascelles & Woods 24). Omission of either vitamin from the mixture reduced synthesis almost to nothing and, in each case, the amount of serine formed was proportional, at limiting concentrations, to the amount of factor present. Glucose, glycine, and formate were also all essential for the synthesis.

Information was next sought as to whether PtG must be converted by the cells to some higher form for activity in this system (Lascelles, Cross & Woods 25). Folinic-acid-SF and _N¹⁰_-formylpteroylglutamic acid had, at most, only equal activity to PtG. On the other hand, it was found that, concurrent with the synthesis of serine in the above system, part of the PtG was converted to a factor (CF) supporting the growth of _Ln. citrovorum_. For production of CF, only glucose, formate, and PtG were necessary. These observations did not, of course, prove that some form of CF was the functional form of folic acid in serine synthesis; they did, however, suggest that the matter should be explored further. An attempt was therefore made to develop a higher form of folic acid within the cells, and then to test them for serine synthesis in the absence of added PtG. Cells first incubated with glucose, formate, and PtG, and then rewashed and tested for serine synthesis, were consistently 40% more active than cells from which formate had been omitted in the first treatment. The higher activity was also obtained with cells first incubated with folinic-acid-SF or _N¹⁰_-formylpteroylglutamic acid without formate.
Cell suspensions of other organisms. Synthesis of serine from glycine and formate has also been obtained with cell suspensions of \textit{Ln. mesenteroides} (M. J. Cross, unpublished material) and of strains of \textit{Saccharomyces cerevisiae} (June Lascelles & P. M. Meadow, unpublished material). These organisms all require PAB (not replaced by PtG) for growth. Cells deficient in PAB were obtained by analogous methods to those used with \textit{Strep. faecalis} R. Synthesis of serine by these cells was negligible unless PAB was added; PtG was inactive.

Identity of serine formed. Throughout this work serine was estimated by microbiological assay with \textit{Ln. mesenteroides} as the test organism. A detailed investigation of conditions affecting its response to serine permitted a satisfactory assay to be developed.

In the case of \textit{Strep. faecalis} R the actual formation of serine has been confirmed by chromatographic methods using “Dowex 50” columns (Moore & Stein \textsuperscript{31}); serine was readily separated from the excess residual glycine.

Carbon dioxide and the synthesis of serine: growth experiments with Leuconostoc mesenteroides. Because of its multiple requirement for amino-acids, \textit{Ln. mesenteroides} is frequently chosen for use in microbiological assay methods. It has an absolute requirement for both serine and glycine. The ability of glycine to replace serine was studied in detail, since it was necessary to devise an assay for serine in the presence of excess glycine. It was found to replace serine only in the presence of (a) PAB, (b) pyridoxal, and (c) an atmosphere enriched with carbon dioxide (CO\textsubscript{2}); formate was inactive (Lascelles & Woods \textsuperscript{25}). When serine was present, PAB was still required though in smaller amounts than for growth on glycine plus CO\textsubscript{2}. Furthermore, with serine present, the addition of thymidine (the basal medium contained purines), permitted growth without PAB, but this was not so for growth on glycine plus CO\textsubscript{2}. These experiments therefore confirmed a specific function of PAB in the conversion of glycine to serine. Steele, Sauberlich, Reynolds & Baumann \textsuperscript{51} have found growth of this organism in the absence of serine to be stimulated by vitamin-B\textsubscript{6}-group factors.

The role of CO\textsubscript{2} in serine synthesis was further investigated (Lascelles, Cross & Woods \textsuperscript{24}). As the atmospheric concentration of CO\textsubscript{2} was increased, the concentration of glycine required to support a given amount of growth decreased. With 5\% CO\textsubscript{2} the concentration of glycine required was about 10 to 20 times that of serine for equivalent growth. The quantitative relationship between glycine and CO\textsubscript{2} is in accord with what might be expected if CO\textsubscript{2} is the ultimate source of the single-carbon fragment for condensation with glycine.

Folinic-acid-SF, but not PtG, replaced PAB for the growth of the organism with serine present. This was also the case for growth on glycine,
but only if pyridoxal was also supplied. As with PAB, a higher concentration of folinic acid was required for growth on glycine than on serine. With folinic acid, however, growth on glycine was no longer dependent upon an atmosphere enriched with CO₂. For quantitative reasons it was impossible that the formyl group of folinic acid itself was acting as the ultimate source of the single-carbon residue. There was always some CO₂ present since the organism was found to produce small amounts during growth on the test medium. A possible interpretation is that the folinic acid acts catalytically, by permitting the efficient utilization of small concentrations of CO₂; alternatively, CO₂ may be concerned, in this organism, with the formation of folinic acid from PAB. 

Conclusions. The experiments with cell suspensions provide direct evidence for a function of the folic-acid group in the synthesis of serine by condensation of glycine (or a derivative) with formate; pyridoxal is also essential for this reaction. There is some evidence that PAB and PtG must be converted to a higher formylated form in order to exert this function. The experiments with growing cultures of _Ln. mesenteroides_ implicate CO₂ in the synthesis of serine but the mechanism is not clear. It may be due to an effect on CF synthesis from PAB. On the other hand, the quantitative relationship between CO₂ and glycine suggests that CO₂ may be an ultimate substrate. In this connexion it is of great interest that serine is the first amino-acid showing isotope activity after brief exposure of actively photosynthesizing algae to ¹⁴C (Calvin ⁴). Recently also Marr & Wilson ²⁹ found that, with a strain of _Brucella_ requiring CO₂ for growth, ¹⁴C from labelled CO₂ was mainly in the glycine/serine fraction of the hydrolysate of the cell protein.

Synthesis of methionine

It is possible here to review only briefly this aspect of the subject. Nutritional studies have implicated this amino-acid more frequently than any other as a product of the function of PAB in cell metabolism. The ability of methionine to overcome sulfonamide inhibition under limited conditions was first reported a decade ago (Bliss & Long; ¹ Harris & Kohn ¹⁶). There is strong evidence that, both with micro-organisms and animal tissues, vitamin B₁₂ is also concerned in the synthesis of this amino-acid.

Intermediates in the synthesis of methionine. Replacement studies with series of biochemical mutants of _Neurospora, Esch. coli_, and _Bacillus subtilis_ indicate the probable path of synthesis of methionine from cysteine to be the condensation of cysteine with homoserine to form cystathionine

---

⁴ Since this paper was read, Mr. M. J. Cross of this laboratory has found that synthesis of CF from PAB by cell suspensions of this organism is considerably increased by CO₂ (unpublished material).
which then undergoes cleavage to yield homocysteine; methionine is finally formed from the latter by methylation (Horowitz; Lampe, Roepke & Jones; Simmonds; Teas; Gots & Koh).

**Role of folic-acid derivatives and vitamin B₁₂ in transformation of homocysteine to methionine.** The inability of homocystine or homocysteine to replace methionine, from the point of view of reducing the amount of PAB required for growth or of overcoming sulfonamide inhibition (Winkler & de Haan; Strehler), suggests that PAB is required for the final methylation of homocysteine. With animal tissues, Dinning, Keith & Day have found that liver and kidney homogenates from folic-acid-deficient chicks have reduced ability to form methionine from homocysteine and choline or betaine. On the other hand, Lampen, Jones & Roepke found that homocystine did partly replace methionine in the mixture of amino-acids which was effective, together with nucleic-acid derivatives, in promoting growth of their PAB-requiring mutant of *Esch. coli*. With rat-liver homogenates, Stekol, Weiss & Weiss have recently found evidence for a function of folic acid in cystathionine metabolism. The folic-acid group may therefore be concerned at more than one stage in methionine synthesis. Earlier work on folic acid and B₁₂ in transmethylation is reviewed by du Vigneaud, Ressler & Rachele.

The role of vitamin B₁₂ in the methylation of homocysteine has also been indicated both in micro-organisms and in animal tissues. Davis & Mingioli obtained a number of mutants of *Esch. coli* which required either B₁₂ or methionine for growth; the latter could not be replaced by homocysteine. Oginsky found that liver homogenate from B₁₂-deficient rats had reduced ability to synthesize methionine from homocysteine and choline or betaine. Young chicks on a B₁₂-deficient diet gave a growth response to methionine, but to homocysteine only if B₁₂ were also given.

More definite evidence for a specific function of these two vitamins in the methylation of homocysteine by bacteria was sought in this laboratory, using simpler systems than whole growing cultures.

**Methionine synthesis by cell suspensions of Escherichia coli.** A number of strains of this organism were found to produce methionine when washed, harvested cells were incubated in homocysteine, glucose, and buffer. Mutant strains requiring PAB and B₁₂, respectively, were available so that deficient cells were readily obtained. This work has been reported briefly by Gibson & Woods.

The B₁₂ mutant, which also responded to methionine, was grown in the presence of that amino-acid in order to obtain vitamin-deficient cells. These failed to synthesize methionine unless B₁₂ was added to the system. At suboptimal concentrations there was a linear relationship between the methionine synthesized and the concentration of B₁₂.
Cells suspensions deficient in PAB were obtained by growth of the PAB mutant in an amino-acid medium containing purines and thymine. Synthesis of methionine occurred only when PAB was added to the suspensions and was further increased threefold by the addition of $B_{12}$. Stimulation of methionine synthesis by $B_{12}$ was not, however, restricted to cells deficient in PAB; it was also found with the parent and other strains. Neither PtG nor folinic-acid-SF replaced PAB, which stimulated synthesis in proportion to its concentration at suboptimal levels.

Even with optimal concentration of $B_{12}$ there was still an absolute requirement for PAB for methionine synthesis by cell suspensions. It seems clear, therefore, that PAB has a function in this reaction quite separate from that of $B_{12}$. It has been suggested (Davis 9), on the basis of the interchangeability of methionine and $B_{12}$ in their ability to spare PAB and to overcome sulfonamide inhibition, that the function of PAB in methionine synthesis is simply due to its requirement for $B_{12}$ synthesis.

In all the above experiments the simple reaction mixture used contained no special source of the methyl group or single-carbon residue, and none of the usual substances was found to enhance methionine formation. Presumably, the organisms provide this residue themselves from the metabolism of the substances present, i.e., glucose and homocysteine. 

**General Considerations**

It is now a generally accepted hypothesis that folic acid (in the widest sense) has finally a co-enzyme function in cell metabolism in reactions involving the transfer of single carbon residues. This is analogous to the now well-proven transfer of two-carbon residues by co-enzyme A (the ultimate co-enzyme form of the growth-factor, pantothenic acid). The hypothesis was first put forward by Shive et al. 6 in connexion with the possible function of PAB in purine synthesis; the more general form is supported by the further work reviewed and reported in this paper.

In each case where evidence has been obtained that the ultimate function of PAB or folic acid is at some specific step in a biosynthetic sequence, the reaction involves the addition of a single-carbon unit (glycine to serine, homocysteine to methionine, carboxamide or derivatives to purines or derivatives). The particular step involved in the synthesis of other amino-acids (e.g., histidine, lysine), and of thymine or thymidine, remains to be determined. In the case of thymine, there is some evidence (page 44) that a diamide may be a precursor; folic acid might therefore again be concerned in the addition of the final carbon atom and ring-closure. In addition, by analogy with methionine, folic acid may function in the provision of the

---

*About the time this paper was read, evidence was obtained (Gibson & Woods 12) that serine acted as methyl donor in this reaction.*
methyl group of thymine, but there is no experimental evidence for this. Elwyn & Sprinso12 found that, with whole rats, the methyl group of thymine can be derived from the \( \alpha \)-carbon of glycine or the \( \beta \)-carbon of serine.

A function of folic acid in enzyme systems required for single-carbon transfer reactions may, of course, include activation of the substrate acting as the single-carbon donor. Dinning, Keith, Davis & Day8 have indeed suggested that, on the basis of aminopterin inhibition, the choline oxidase enzyme system of chicken marrow contains folic acid. This enzyme is thought (Dubnoff10) to render labile the methyl group of choline.

The chemical nature of the ultimate co-enzyme form of folic acid is still obscure. The recently discovered formyl derivatives of tetrahydropteroylglutamic acid do not have the desired properties as far as can be judged from experiments with whole cells. It is an attractive hypothesis that the reversible formylation of folic acid is the actual mechanism of the function of single-carbon transfer; if folinic acid were the actual co-enzyme it would then be expected that the unsubstituted tetrahydro-PtG would be equally effective. Broquist et al.2 tested this substance but found it considerably less active than folinic acid, though more active than PtG, in supporting the growth of Ln. citrovorum, and in overcoming the toxic action of aminopterin in mice. The co-enzyme form of folic acid may be some still more complex molecule containing a folinic-acid residue.

The interrelationship of vitamin B\(_{12}\) and folic acid in cell metabolism is not clear. Both appear to be concerned in the synthesis of purine and pyrimidine deoxyribosides, and in the methylation of homocysteine to methionine. The balance of present evidence suggests that the two factors have separate functions in these reactions, and that the effect of folic acid (or of PAB) is not merely due to its probable role at some stage in the synthesis of vitamin B\(_{12}\).

---

**SUMMARY**

Vitamin-B-group substances are required by all types of living cells for their normal metabolism and growth, and this common requirement necessarily reflects a fundamental similarity in basic cell-processes. These substances appear to function as components of catalytic systems, and many have been found to form part of the structure of co-enzymes or prosthetic groups of important enzyme systems.

---

**RÉSUMÉ**

Tous les types de cellules vivantes on, besoin pour se développer normalement des substances vitaminiques du groupe Bt ce qui indique une similitude profonde dans les processus cellulaires élémentaires. Ces substances agissent comme compo- sants de systèmes catalytiques, et plusieurs d'entre elles font partie des groupes prothétiques de systèmes enzymatiques importants. Des études sur la nutrition
Studies of bacterial nutrition and metabolism have yielded valuable information on the function of these growth-factors, particularly in the case of those B-vitamins which are ultimately concerned with synthetic rather than catabolic processes. In this paper, the methods of investigation followed in such studies, and some of the results obtained, are discussed.

The four methods of approach which have proved fruitful are: (1) studies of bacterial nutrition in which (a) the replacement of the requirement for a B-vitamin by a substance of a different chemical type, and (b) the inhibition of the utilization of a B-vitamin by a chemical analogue of a growth-factor were investigated; (2) study of the metabolism of organisms deficient in a given vitamin; (3) study of the metabolism of the vitamin itself; and (4) investigation of the replacement of co-enzymes.

Detailed reference is made to work on the functions of p-aminobenzoic acid (PAB), of the folic-acid group of growth-factors—folic acid (pteroylglutamic acid (PtG)); folinic acid (tetrahydroformylpteroylglutamic acid); etc.—and of vitamin B₁₂.

The discovery that PAB is able to overcome the inhibition of bacterial growth by the sulfonamide drugs led to the hypothesis that PAB is an essential metabolite for micro-organisms, and that its utilization by the cell is competitively inhibited by the sulfonamides because of their similarity in chemical structure. The further discovery that PAB is an essential growth-factor for a wide variety of micro-organisms led to a desire for more information on its function in the bacterial cell.

Investigations have indicated that the chief function of PAB in the cell is for the synthesis of folic acid and related substances which, in turn, are essential metabolites for bacteria, and that this synthesis is inhibited by the sulfonamides.

The exact relationship between PAB, PtG, and folinic acid is not yet clear, but it is possible that both PtG and folinic acid are converted by the organism into intermediates in the formation of the final functional co-enzyme form of folic acid from PAB. Some organisms, however, and le métabolisme bactériens ont donné d’utiles renseignements sur la fonction de ces facteurs de croissance, surtout en ce qui concerne celles des vitamines B, qui interviennent plutôt dans les synthèses que dans les réactions cataboliques. Les méthodes de recherche adoptées pour ces études sont décrites par l’auteur et certains résultats sont discutés.

Quatre types de recherches ont donné des résultats intéressants: 1) les études de nutrition bactérienne portant sur a) le remplacement de la substance du groupe B, nécessaire, par une substance d’un autre type chimique, b) l’inhibition de l’utilisation d’un composé du groupe B par une substance chimique analogue au facteur de croissance; 2) l’étude du métabolisme des organismes carencés en vitamine d’un type donné; 3) l’étude du métabolisme de la vitamine elle-même; 4) le remplacement des co-enzymes.

Le rôle de l’acide p-aminobézoïque (PAB), des facteurs de croissance du groupe de l’acide folique — acide folique (ptéroylglutamique PtG) acide folinique (tétrahydroformylptéroylglutamique) — et celui de la vitamine B₁₂ sont étudiés en détail.

A la suite de la découverte de la suppression de l’effet inhibiteur des sulfamides par PAB, on a supposé que PAB était un métabolite microbien important et qu’il y avait compétition entre PAB et sulfamide dans la cellule, en raison d’une similitude de structure chimique. Le rôle de facteur de croissance joué par PAB pour des micro-organismes nombreux et variés a justifié des recherches plus approfondies sur ses fonctions dans la cellule bactérienne. Elles ont montré que PAB joue un rôle essentiel dans la synthèse de l’acide folique et des substances apparentées et que cette synthèse est inhibée par les sulfamides.

La relation exacte entre PAB, l’acide ptéroylglutamique (PtG) et l’acide folinic n’est pas encore clairement établie, mais il est possible que ces deux derniers corps, transformés, jouent le rôle d’intermédiaires dans la formation de l’acide folique — fonctionnant comme coenzyme — à partir
may not be capable of effecting such conversions.

Replacement and inhibition studies have both provided strong evidence that PAB and the folic-acid group of substances are concerned in the synthesis of:

(a) nucleic-acid derivatives, such as purines and thymine or, more probably, thymidine; and

(b) certain amino-acids, particularly methionine and serine.

In the case of (a), the evidence has been confirmed by the fact that accumulation of 4-aminoimidazole-5-carboxamide (a possible precursor of purines) occurs in cultures partially inhibited with either sulfonamides or aminopterin (an analogue of PtG), and that growth of Lactobacillus casei in media deficient in PtG leads to cells deficient in desoxyribonucleic acid.

With regard to (b), experiments have recently been carried out in the author’s laboratory to obtain more direct evidence of the function of the B-vitamins in the synthesis of serine and more information as to the nature of the particular stage at which they function.

Washed cell-suspensions of Streptococcus faecalis R were found to synthesize serine when incubated in a medium containing glycine, formate, and glucose. Cells deficient in PtG and pyridoxal (vitamin B6), however, did not synthesize serine unless these vitamins were added to the reaction mixture. The synthesis of serine is accompanied by the conversion of PtG to folic acid—a process for which the presence of both glucose and formate is required. Deficient cells first incubated in glucose, formate, and PtG synthesize serine more actively (when tested in the usual system but omitting PtG) than cells similarly treated in the absence of formate.

Similar experiments with cell suspensions of Leuconostoc mesenteroides have shown that pyridoxal and PAB are also required de PAB. Cependant, il se peut que certains organismes ne soient pas capables d’effectuer cette transformation.

Les études relatives au remplacement et à l’inhibition ont montré à l’évidence que PAB et les substances du groupe de l’acide folique sont impliquées dans la synthèse

a) des dérivés de l’acide nucléique, tels que les purines et la thymine — ou plus probablement la thymidine ;

b) de certains amino-acides, en particulier la méthionine et la sérine.

Dans le cas de a), la preuve a été renouvelée par les constatations suivantes : dans les cultures partiellement inhibées par les sulfamides ou l’aminoptérine (analoge de PtG), il se produit une accumulation de amino-4 imidazole carboxamide-5 (précurseur éventuel des purines) ; la culture de Lactobacillus casei dans des milieux carencés en PtG donne des cellules carencées en acide désoxyribonucléique.

Quant à b), des expériences récentes ont été effectuées dans le laboratoire de l’auteur pour déceler la fonction des vitamines B dans la synthèse de la sérine, et le stade auquel elles sont actives.

Une suspension de cellules lavées de Streptococcus faecalis R ont synthétisé la sérine dans un milieu contenant de la glycine, du formiate et du glucose. Les cellules carencées en PtG et en pyridoxal n’ont synthétisé la sérine que lorsque ces deux vitamines ont été ajoutées au milieu. La synthèse de la sérine est accompagnée de la conversion de PtG en acide folinique, processus qui exige à la fois la présence de glucose et de formiate. Des cellules carencées cultivées préalablement en présence de glucose, de formiate et de PtG synthétisent la sérine plus activement (lorsqu’elles sont étudiées dans les conditions habituelles mais sans PtG) que les cellules cultivées de la même façon, mais sans formiate.

Des expériences analogues avec des suspensions de Leuconostoc mesenteroides ont montré que le pyridoxal et PAB sont
for serine synthesis by this organism. It was found, however, that the serine requirement for growth of *Ln. mesenteroides* could be replaced by relatively high concentrations of glycine, provided that the atmosphere was enriched with carbon dioxide; the carbon dioxide could be replaced by folinic acid, but not by either formate or PtG.

Investigations on the synthesis of methionine from homocysteine, in the presence of glucose, by PAB-deficient cell suspensions of a PAB-requiring mutant of *Escherichia coli* have indicated that the synthesis is dependent on the presence of PAB and is greatly stimulated by vitamin B₁₂.

From the evidence now accumulated it seems likely that the functional co-enzyme form of PAB and folic acid is part of an enzyme system which activates certain substrates to donate single-carbon units to an acceptor.

As yet, little information is available concerning the function of vitamin B₁₂ in micro-organisms, though replacement studies have indicated a general role in the synthesis of purine and pyrimidine desoxyribosides, and the fact that the growth requirement for B₁₂ of an *Esch. coli* mutant can be met by methionine suggests a function in methionine synthesis.

It is probable, therefore, that both folic acid and B₁₂ are involved, though presumably in different reactions, in the biosynthesis of nucleic-acid derivatives and of methionine.

nécessaires à la synthèse de la sérine par cet organisme. Cependant, la sérine exigée par *Ln. mesenteroides* pour sa croissance peut être remplacée par la glycine, à concentration relativement élevée, à condition que l’atmosphère soit enrichie en CO₂; ce dernier peut être remplacé par l’acide folinique; mais ni le formiate ni PtG ne peuvent lui être substitués.

Des recherches sur la synthèse de la méthionine à partir de l’homocystéine en présence de glucose, par des suspensions de cellules carencées en PAB d’un mutant de *Escherichia coli*, exigeant le PAB, ont montré que la synthèse dépend de la présence de PAB et qu’elle est stimulée par la vitamine B₁₂.

Il semble d’après ces résultats que PAB et l’acide folique fonctionnant comme co-enzyme font partie d’un système enzymatique qui active, dans certains substrats, la libération d’unités à un atome de carbone, et leur transfert sur un accepteur.

Jusqu’à maintenant, la fonction de la vitamine B₁₂ chez les micro-organismes est encore peu connue; cependant des études de remplacement permettent de penser qu’elle joue un rôle dans la synthèse des désoxyribosides puriques et pyrimidiques. Le fait que l’exigence de B₁₂ par un mutant de *Esch. coli* peut être satisfaite par la méthionine suggère que B₁₂ joue un rôle dans la synthèse de cette substance.

Il est probable, en conséquence, que l’acide folique et la vitamine B₁₂ sont impliqués tous deux dans la biosynthèse des dérivés de l’acide nucléique et celle de la méthionine.

REFERENCES

7. Davis, B. D. & Mingioli, E. S. (1950) *J. Bact.* 60, 17
16. Harris, J. S. & Kohn, H. I. (1941) J. Pharmacol. 73, 383
34. Peters, R. A. & Thompson, R. H. S. (1934) Biochem. J. 28, 916
47. Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M. & Sutherland, J. E. (1948) J. Amer. chem. Soc. 70, 2299
54. Strehler, B. L. (1950) J. Bact. 59, 105
58. Winkler, K. C. & Haan, P. G. de (1948) *Arch. Biochem.* 18, 97