ANALYTICAL REVIEW

The Interrelationships of Factors that Influence the Megaloblastic Anemias


Several substances are capable of producing a clinical and hematologic response in patients suffering from megaloblastic anemia. Those that have so far been shown to be of most clinical importance are vitamin B₁₂, pteroylmonoglutamic acid (possibly folic acid), and the Leuconostoc citrovorum factor (probably folic acid). The interrelationships of these hemopoietic agents are as yet uncertain, and in this necessarily complicated review we can only examine the evidence derived from many investigations without being able to weld the theories into one satisfactory master plan. Many of these theories are based on microbiologic or animal experiments and caution has to be exercised in applying the results of such investigations to errors of human metabolism.

Little need be said about the historical aspects of the subject. The experiments of Castle and his co-workers, and the resulting theory that the anemic principle of liver was formed by the interaction of extrinsic factor from the food and intrinsic factor secreted by the normal stomach are well known. The synthesis of pteroylglutamic acid was followed by many reports of its effectiveness in the treatment of the megaloblastic anemias, and soon afterwards Rickes et al., in the United States and Lester Smith in Great Britain announced that they had isolated from liver a highly potent red pigment that appeared to be the "specific anemic factor." In 1948, Sauberlich and Baumann reported the presence in liver of a growth factor for the streptococcus Leuconostoc citrovorum, and this factor has been shown to be effective in the treatment of pernicious anemia in relapse. Bond et al. have prepared from liver a substance that they have named folic acid. This appears to have properties similar to those of the Leuconostoc citrovorum factor and may be identical with it. In this review the term citrovorum factor will be used. A synthetic form of this factor has recently been prepared from pteroylglutamic acid by Brockman et al.

It should be stated that the preparation used in this Department and already reported on was the synthetic form and not a preparation of biologic origin as we had understood. The isolation and preparation of these various substances has reopened the question of the metabolic faults in the megaloblastic anemias.

Microbiologic Interrelationships and Their Applications

Certain vitamins and amino acids may be measured quantitatively by microbiologic assay, the substance concerned acting under the conditions of the assay as a growth factor for a suitable strain of micro-organism. This type of assay is possible with vitamin B₁₂, pteroylglutamic acid and the citrovorum factor.

Pteroylglutamic acid. Suitable test organisms for pteroylglutamic acid are Lactobacillus casei and Streptococcus fecalis R.

Interpretation of the results of microbiologic assay for pteroylglutamic acid are compli-
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cated by the fact that this synthetic substance is one of a family of compounds that vary in their capacity for maintaining the growth of these organisms. The synthetic pteroylglutamic acid contains only one glutamic acid residue and corresponds to the L. casei factor isolated from liver. There is also a fermentation L. casei factor which has been isolated from the aerobic culture of an organism of the genus Corynebacterium. This "fermentation form" contains three glutamic acid residues in the side chain. It has been synthesized and is available commercially as Teropertin. It is believed, however, that the conjugate present in yeast and in foodstuffs is largely pteroylhexaglutamylglutamic acid (pteroylheptaglutamic acid), a gamma-linked peptide, the exact formula of which is uncertain. This conjugate has not been synthesized. The fermentation factor (pteroyltetraglutamic acid) is active for the growth of L. casei, but relatively inactive for S. fecalis. The naturally occurring hexaglutamyl conjugate is inactive for L. casei and for S. fecalis. In addition there has been synthesized the conjugate pteroyl-alpha-glitamylglutamic acid (pteroyldiglutamic acid) which is inactive for L. casei and S. fecalis, and does not occur in nature. In metabolic studies, it is usual to release free pteroylglutamic acid from its conjugate forms by enzymatic digestion or by other means before microbiologic assay is performed.

Moreover, other substances exist which will support the growth of these test organisms, and the most important in relation to the subject of this review would appear to be thymine, thymidine (thymine desoxyriboside), and the citrovorum factor.

It has been shown by Snell and Mitchell and by Stokstad that thymine and purines will support the growth of S. fecalis and L. casei in the absence of pteroylglutamic acid.

Stokes who could not demonstrate folic acid in organisms grown in a medium containing thymine in place of folic acid suggested that folic acid functions as a co-enzyme for the enzyme system responsible for the synthesis of thymine or a thymine-like compound which, in turn, is used by bacteria to form nucleic acid. In the presence of thymine, folic acid is no longer necessary for growth. The synthesis of thymine becomes superfluous in the presence of an extraneous supply of the substance. Hall, however, showed synergistic growth-promoting effects of folic acid and thymine on S. fecalis R, and considered that it seemed likely that thymine might actually be a precursor of folic acid or that thymine was participating in some alternative metabolic path.

Rogers and Shive found that, in the presence of purines, thymine prevented the growth inhibiting effects of methylfolic acid on L. casei and agreed with Stokes that folic acid functions in the biosynthesis of purines and thymine or their equivalents.

Turning to animal experiments, we find that Petering and Delor could not substitute thymine or thymine together with adenine sulfate or adenosine for folic acid in promoting growth and a rise in white cell count in folic acid-deficient rats, and that Daniel et al. could not substitute thymine for folic acid in chick growth experiments.

Wright, Skeggs and Huff have carried the theory of Stokes further by suggesting that vitamin B₁₂ may function as a co-enzyme in carrying out reactions concerned with the conversion of thymine to thymidine. In their view, then, transferring bacteriologic findings into the field of clinical hematology, the primary biochemical defect in pernicious anemia is an inability to synthesize certain nucleosides, particularly thymidine, from parent purines or pyrimidines.

From consideration of bacteriologic experiments and from their own results in patients with pernicious anemia and related megaloblastic anemias, Vilter
et al.\textsuperscript{27} have put forward the following hypothesis to explain the defects in various types of megaloblastic anemia. Folic acid, possibly in conjunction with another factor or factors, acts as a co-enzyme (or as a substrate in the formation of another co-enzyme) concerned with the formation from precursors such as uracil, of purines and thymine or their equivalents. Vitamin B\textsubscript{12} then activates the formation of nucleosides (thymidine) from the purines and pyrimidines, and from the thymidine are formed nucleotides and hence nucleic acid. To bring this into line with recent work on the citrovorum factor, it would be necessary to postulate that the hematopoietically active co-enzyme in the first step is not pteroylglutamic acid, but citrovorum factor. This may be represented as follows:

\begin{tikzpicture}[->,auto, node distance=1.5cm, on grid]
  \node (A) {NH\textsubscript{2} & C sources \rightarrow Purines & Pyrimidines};
  \node (B) [below right of=A] {Citrovorum Factor \rightarrow Ribose & Desoxyribose \rightarrow Nucleosides \rightarrow Nucleotides \rightarrow Nucleic Acid (Thymidine)};
  \node (C) [below of=B] {Folic Acid \rightarrow Nucleic Acid (Thymidine)};
  \node (D) [below of=C] {Vitamin B\textsubscript{12}};

  \path (A) edge (B)
  (B) edge (C)
  (C) edge (D);
\end{tikzpicture}

This hypothesis is very theoretical and much further clinical work is necessary before it can be credited or disproved. For example, we know nothing of the effects of thymidine in megaloblastic anemia of pregnancy or in certain other forms of megaloblastic anemia that are refractory to vitamin B\textsubscript{12} therapy, conditions which Vilter and his associates suggest may be due to a defect in the formation or utilization of thymine and perhaps other pyrimidines and purines. We know that thymine has hematopoietic activity in large doses in pernicious anemia and sprue,\textsuperscript{31} and that it will induce a response in pernicious anemia even after pteroylglutamic acid has lost its effect in a dosage of 30 mg. three times a week.\textsuperscript{27} It has been shown that pteroylglutamic acid has a typical mass action effect in that when hematologic relapse occurs, an increase in the dose will induce another temporary remission. It is possible that thymine, too, is effective as a result of mass action. Vitamin B\textsubscript{12} may be inactive in certain forms of megaloblastic anemia because, since certain earlier links in the chain are absent, vitamin B\textsubscript{12} cannot play its part in activating the formation of nucleosides. Hausmann\textsuperscript{29} has reported responses in pernicious anemia to doses of 1 to 2 Gm. of thymidine. The necessity for administering such large doses of thymidine in pernicious anemia does not invalidate this theory, since in normal metabolism the thymidine might be released in small quantities in the environment of the hemopoietic tissue whereas when it is given by mouth or by injection it is disseminated throughout the body as a whole.

Similar types of chain reaction involving vitamin B\textsubscript{12} may be concerned with the integrity of the nervous system and the metabolism of bilirubin.\textsuperscript{37}

In addition to explaining the various clinical findings by this or any other theory, account must be taken of the cytochemical changes found in the megaloblastic anemias. In pernicious anemia there is an increase of desoxyribosenucleic acid in the nucleus, and a great increase of ribonucleic acid in the cytoplasm of the red cell precursors.\textsuperscript{29}
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Vitamin B₁₂

Suitable test organisms that have been used for the microbiologic estimation of vitamin B₁₂ are *Lactobacillus lactis* Dorner, *Lactobacillus leichmannii*, *Euglena gracilis* and a mutant strain of *Escherichia coli*.

As with pteroylglutamic acid, there is the difficulty that under assay conditions other substances may replace vitamin B₁₂ for the growth of the test organisms, but this may be overcome by destroying the vitamin B₁₂ by alkaline hydrolysis and re-estimating the content of growth factors for the test organism. Thymine is one such substance. To add to the complications, there have recently been isolated forms other than vitamin B₁₂ itself. These are known as vitamin B₁₂α, B₁₂β (which is probably the same as B₁₂α), B₁₂γ and B₁₂δ. The complexities of the chemistry of the B₁₂ group of vitamins cannot be considered in a review of this nature, but it is known that vitamin B₁₂ contains a cyanide group which is replaced by a hydroxy group in vitamin B₁₂α, and by a nitrite group in B₁₂β. Vitamin B₁₂δ is produced when the nitrite group is removed from B₁₂β. In microbiologic assays, the practical importance of the absence of the cyanide group in B₁₂α, B₁₂β and B₁₂δ is that the various forms are now believed to differ in microbiologic activity, but as yet they have not been shown to differ in clinical activity, whereas the addition of sodium cyanide to the assay medium would appear to convert the other forms to vitamin B₁₂. The latter form is more active for the growth of *Lactobacillus lactis* Dorner and *Lactobacillus leichmannii*. Hence there may be an increase in apparent vitamin B₁₂ content of the substance or fluid being tested with these organisms. This discrepancy is believed not to occur if *Escherichia coli* is used as the test organism. Any metabolic studies involving the microbiologic assay of vitamin B₁₂ must be interpreted in the light of these findings.

Unfortunately there are further difficulties, in that vitamin B₁₂ may occur in “bound” forms that cannot be measured microbiologically. It has been shown by Ternberg and Eakin that vitamin B₁₂ may be rendered microbiologically unavailable if incubated with normal gastric juice. Microbiologically inactive forms have also been shown to be present in the serum and in the feces. Such microbiologically inactive forms may be released by enzymatic digestion, by autolysis or by heating, but some such forms are released by heating to 100 C. and not by digestion with pancreatin. There is thus the further difficulty in relation to metabolic studies that vitamin B₁₂ may be present in forms not available to test organisms. This may be extremely important in relation to tests on liver and liver extracts, where it has been suggested that vitamin B₁₂ may be present in some conjugate form from which it may be released by proteolysis or by the addition of KCN.

Leuconostoc Citrovorum Factor

Reference has already been made to the isolation of the citrovorum factor from liver. We have seen, too, that in a folic acid free medium the citrovorum factor may replace pteroylglutamic acid as a growth factor for *L. casei* and for *Streptococcus fecalis R*. Accordingly many estimations of the folic acid content of substances or fluids have been incorrect since citrovorum factor was included as pteroylglutamic acid. A correction for this may be made by estimating the
combined pteroylglutamic acid and citrovorum factor content using *S. fecalis* as test organism and then estimating the citrovorum factor content separately using *Leuconostoc citrovorum*. The citrovorum factor is unstable, and even mild acid treatment will convert it to pteroylglutamic acid. Metabolic studies of pteroylglutamic acid must take consideration of the possibility that citrovorum factor is also present and being measured. Moreover, thymidine will stimulate the growth of *Leuconostoc citrovorum*, as will pteroylglutamic acid in the presence of purines. Fortunately, however, pteroylglutamic acid must be in high concentration and is usually eliminated by simple dilution. Thymidine and citrovorum factor will inhibit the toxic effects for bacteria of folic acid antagonists. This will be referred to later in connection with animal experiments.

The microbiologic studies of Sauberlich and of workers at the Lederle Laboratories have led to the following conclusions about the relationships of vitamin B12, pteroylglutamic acid, citrovorum factor and thymine in the nutrition of *L. leichmannii* and *Leuconostoc citrovorum*. If pteroylglutamic acid is present *L. leichmannii* can produce citrovorum factor; if vitamin B12 or thymidine is added to the medium this lactobacillus can produce deoxyribosides of guanine, hypoxanthine, adenine and cytosine. *Leuconostoc citrovorum*, however, can synthesize its own vitamin B12 from precursors, and, under the influence of this vitamin B12, can synthesize deoxyribosides of guanine, hypoxanthine, adenine and cytosine. *Leuconostoc citrovorum* can make thymidine only if citrovorum factor is supplied. It appears therefore that citrovorum factor is a catalyst concerned with the formation of thymidine.

Recently, it has been found that cortisone will replace pteroylglutamic acid for *S. fecalis* and the citrovorum factor for *L. citrovorum*. It is obvious that the interrelationships of these various factors for bacterial metabolism are complex, and it is likely that equal complexities attend their function in the human body.

**Chemical Interrelationships**

In 1941, Mitchell, Snell and Williams prepared from spinach leaves a growth factor for *L. casei* and *S. fecalis* which they called folic acid, and the terms folic acid and pteroylglutamic acid are now used synonymously by most authors including the author of the present review. This may not be quite correct, however, since folic acid itself has not been isolated in the pure state and it has been shown that a folic acid concentrate prepared from spinach exerts a synergistic action on synthetic pteroylglutamic acid. This matter is discussed further by Robinson.

Vitamin B12 (and the forms B12a, B12c) are not related chemically to pteroylglutamic acid or to the citrovorum factor. This last substance has very recently been reported to be 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid.

Numerous papers have been published recently on the chemistry of vitamin B12, largely by workers at British Drug Houses and Glaxo Laboratories in Great Britain and at Merek and Co. in the United States. Brink et al. who showed that vitamin B12 exists as a cyano-cobalt coordination complex, suggested that cobalamin should be the name given to the part of the B12 molecule attached to the cyano group. Thus vitamin B12 can be called cyanocobalamin, and B12c becomes hydroxocobalamin. Cooley et al. suggest that "cobalamin" is stored by the tissues in a cobalichrome type of combination with a compound of pro-
tein or polypeptide character. These authors suggest that, in the body, cobalamin performs its functions by reversible release of cyanide which then becomes available for inhibiting an associated system of enzymes, as follows:

\[
\text{Depot Form} \xrightarrow{\text{Enzyme}} \text{CN} \xrightarrow{\text{B}_{12b}} \text{B}_{12} \xrightarrow{\text{Reduction by}} \text{substrate with inhibition}
\]

**Bone Marrow Culture**

It is possible to culture bone marrow cells without using a very elaborate technic, and Rusznęk, Löwinger and Lajtha have demonstrated in the serum of patients with untreated pernicious anemia a factor that inhibits the conversion of megaloblasts to normoblasts in cultures of this nature. This finding has been confirmed and Lajtha has shown that the cerebrospinal fluid in pernicious anemia has a similar inhibiting effect. He has also found that pteroylglutamic acid has a significant ripening effect on megaloblasts under culture conditions, whereas vitamin \( B_{12} \) has no such effect. After admixture with a source of intrinsic factor the latter vitamin will induce maturation whereas gastric juice itself is ineffective. This is in contrast to the microbiologic activity of these substances since Ternberg and Eakin have shown that intrinsic factor makes vitamin \( B_{12} \) nonavailable for the growth of *L. leichmannii*. Citrovorum factor will induce maturation of megaloblasts in culture.

Using a more complicated culture technic, Hays found that pteroylglutamic acid and pteroylhexaglutamylglutamic acid did not cause maturation of primitive red cells of the rat marrow, whereas rat serum, normal human serum and liver extract gave such maturation. The serum from a pernicious anemia patient in relapse appeared to lack the maturation factor. The conditions of this investigation were, however, more artificial than was the case with those referred to above.

Lajtha's original hypothesis based on his earlier experiments is that pteroylglutamic acid acts directly on the cells in vitro and in vivo without the help of "liver factor" or vitamin \( B_{12} \), and that the activity of folic acid normally present is inhibited by a factor present in the serum and cerebrospinal fluid. The effect of this inhibitory factor is normally counteracted by the active liver principle. He suggests further that vitamin \( B_{12} \) has to be transformed by the organism to the active hematopoietic factor.

This theory has to be reinterpreted in the light of Callender and Lajtha's later experiments, but the findings would suggest that the intrinsic factor converts vitamin \( B_{12} \) into the active form. It should be recollected that Ross has shown that in pernicious anemia, vitamin \( B_{12} \) after injection appears to circulate in a form that is not available for the growth of micro-organisms unless treated by heating. Comparison of this approach to the problem of the interrelationship of the hematopoietic factors with that of the microbiologist and clinician requires further studies involving the use of uracil, thymine and thymidine in bone marrow culture studies.

Another method of investigating the action of the hemopoietic factors upon
bone marrow cells is that employed by Horrigan, Jarrold and Vilter \(^\text{27}\) who instilled pteroylglutamic acid, vitamin \(B_12\) and citrovorum factor directly into the marrow cavity of pernicious anemia patients in relapse.

They reported results opposite to those of Lajtha in that vitamin \(B_12\) acted upon the marrow cells, whereas citrovorum factor was ineffective in the dosage used, as was pteroylglutamic acid. They stated further that there is abnormal ribonucleic acid in the cytoplasm of the primitive red cells in pernicious anemia, and suggested that in this condition, hemoglobin synthesis begins while the ribose polynucleotide contents of the cells is still high.

**Interrelationships in Animal Experiments**

Here again the literature is vast and complex and much of it has been summarized in a previous review. \(^\text{73}\) In certain animals, particularly the pig, it has been found possible to produce a megaloblastic form of anemia by dietetic means, by the use of folic acid antagonists, or both.

In a recent paper, Cartwright and his co-workers \(^\text{74}\) have extended their work on macrocytic anemia in swine. Anemia was produced by the administration of a purified diet containing casein and supplemented with seven vitamins, sulfasuxidine and a crude methyl folic acid antagonist. Control animals received folic acid. There was a slight and definitely suboptimal hematopoietic response following the administration of crystalline vitamin \(B_12\), proteolysed liver, marmite, crude desoxyribosenucleic acid and crude ribonucleic acid. The administration of thymine in a dosage of 100 Gm. in ten days, in addition to a crystalline vitamin \(B_12\) did not appreciably augment the activity of the vitamin. Histidine was tried because Hall \(^\text{75}\) has reported that it is a biologic precursor of folic acid but there was no consistent response to this. The investigators consider that the experimental anemia corresponds to the pteroylglutamic acid-responsive, liver-refractory megaloblastic anemia in the human, such as nutritional megaloblastic anemia, megaloblastic anemia of pregnancy and "refractory megaloblastic anemia" or achrastic anemia. It is of interest that, in the pigs, proteolysed liver was more active than marmite, although this latter autolysed yeast preparation was found to contain seven to eight times more folic acid than did proteolysed liver by microbiologic assay. This difference in hematopoietic activity might be due, as suggested previously, \(^\text{14}\) to an as yet undiscovered antianemic principle.

Experiments with monkeys are complicated by the possible existence of Wills' factor. \(^\text{73, 76}\) This factor is believed to occur in autolysed yeast preparations, which contain about 1.8 mg. of growth factors for \(S. \text{f}e\text{c}a\text{l}i\) per ounce. Whether Wills' factor is merely folic or folinic acid has not yet been satisfactorily established.

Smaller animals such as the chick, rat, mouse or turkey have been used for measuring the effect of the various hematopoietic factors on growth, but are less suitable for following changes in the marrow and peripheral blood. Work dealing with the relationship of vitamin \(B_12\) to the animal protein factor, or the mode of action of aureomycin in promoting growth in animals \(^\text{79}\) is outside the scope of this review.

Theories of the role of citrovorum factor in the interrelationships of the hematopoietic factors is, however, based largely upon animal and microbiologic experiments.

When 4-aminopteroylglutamic acid or methylfolic acid is added to the medium, growth of \(Leu\text{conos}t\text{oe} \text{citr}\text{ov}oru\text{r}um\) is inhibited or depressed. Citrovorum factor concentrates were prepared from liver extracts and from the urines of rats fed
folic acid, and these concentrates prevented the inhibitory effect of the folic acid antagonist. High concentrations of pteroylglutamic acid were weakly effective and vitamin B₁₂ was ineffective against 4-aminopteroylglutamic acid toxicity. Thymidine was less effective than citrovorum factor, the response being variable and occurring after a longer incubation period.

It has also been shown that citrovorum factor will reverse the inhibitory effects of 4-aminopteroylglutamic acid for *Escherichia coli*, and for *S. fecalis*.

Cortisone too has this effect.

In an important paper, Nichol and Welch have shown that rat liver slices incubated in Krebs-Ringer phosphate solution will form from synthetic pteroylglutamic acid a factor that supports the growth of *Leuconostoc citrovorum*, and that this conversion is inhibited by 4-aminopteroylglutamic acid. When rats are given pteroylglutamic and 4-aminopteroylglutamic acid, there is again an inhibition of the synthesis of citrovorum factor as measured by urinary excretion. Moreover, the inhibitory effects of 4-aminopteroylglutamic acid on the growth of rats may be overcome by citrovorum factor and not by pteroylglutamic acid.

In mice, too, Broquist et al. have found that citrovorum factor will prevent the toxic effect of 4-aminopteroylglutamic acid, and citrovorum factor will substitute for pteroylglutamic acid in promoting the growth of chicks on a folic acid deficient diet.

It appears likely, therefore, that pteroylglutamic acid is converted in the animal to citrovorum factor, and that this step is prevented by 4-aminopteroylglutamic acid. If this is true in the human, then citrovorum factor would function metabolically at a later stage than pteroylglutamic acid, but it will be some time yet before we shall know whether or not citrovorum factor has a mass action effect and acts as a co-enzyme in thymine synthesis, or whether there is evidence that the action of citrovorum factor is at a late stage in the chain of nucleic acid anabolism. Citrovorum factor should be of value in preventing or treating some of the toxic effects of aminopterin treatment of acute leukemia.

*Studies in the Human*

There is now widespread agreement with the view that the extrinsic factor is in fact vitamin B₁₂ or related substances, that in some way the intrinsic factor promotes its absorption, and that vitamin B₁₂ perhaps together with some of its modifications and conjugated forms is the "specific antianemic factor" of liver extract. It is not certain whether or not normal gastric juice combines with vitamin B₁₂ stoichiometrically as in the in vitro investigations of Ternberg and Eakin or whether their apoerythrin is the same thing as intrinsic factor. Since other reviews deal with the absorption of vitamin B₁₂, its existence in the diet, and its possible synthesis or destruction by bacteria in the intestinal lumen, these matters will not be referred to in detail. The use of antibiotics as therapeutic agents in the megaloblastic anemias would also appear to be related to the action of bacteria in the intestine.

One of the most important practical clinical questions and one pertinent in this review, is whether or not vitamin B₁₂ is as satisfactory as liver extract by
injection, in the maintenance treatment of pernicious anemia. This has not yet been decided, but most clinicians now believe that such is the case.

Most patients feel as well in general health with vitamin B₁₂ as with liver injections, and the beneficial effects on neurologic complications are at least as satisfactory with vitamin B₁₂. There have been reports of the persistence of macrocytosis⁸⁸ and of subnormal prothrombin values in the blood⁹⁹ in patients treated with vitamin B₁₂, but as yet these findings have not been confirmed. Further time must elapse before we can say with absolute certainty that the therapeutic effect of refined liver extracts is due entirely to vitamin B₁₂. Of the various substances already mentioned, there is evidence that a hematologic remission may be induced in pernicious anemia with liver, liver injections, vitamin B₁₂ and its various modifications, proteolyzed liver, desiccated hogs' stomach by mouth and at least partial remissions from pteroylglutamic acid, citrovorum factor, uracil, thymine and thymidine. Trials of autolyzed yeast preparations have given variable results. While most patients with pernicious anemia will respond at first to pteroylglutamic acid, with continued therapy hematologic, lingual and neurologic relapses occur. On the other hand, megaloblastic anemias refractory to vitamin B₁₂ may respond to pteroylglutamic acid or citrovorum factor.

To the clinical investigator the problem of the megaloblastic anemias is very largely the problem of explaining how it is that all these different substances can produce an erythrocyte response and whether they act through parallel reactions or as various links in the same chain. The pediatrician has found, moreover, that there is some relationship between a low ascorbic acid intake and folic acid deficiency in megaloblastic anemia of infancy.⁹⁰

In addition to these matters, an explanation has to be found in pernicious anemia for the presence of a high blood phenol level with an increased urinary excretion of total phenolic compounds. There is evidence that liver or stomach preparations⁹¹ or vitamin B₁₂ ⁹² will decrease this abnormal urinary excretion of total phenols by a reduction in the phenolic fraction containing the hydroxyphenyl acids considered to be metabolic intermediates derived from tyrosine. This may be an indication that tyrosine or a tyrosine derivative plays a part in erythrocyte development, or merely that vitamin B₁₂ has an effect on the metabolism of amino acids in general.

It has been shown by several workers that the urines of pernicious anemia patients contain small amounts of pteroylglutamic acid and that the quantity does not differ greatly from what is found in controls.⁴¹, ⁹³, ⁹⁴ On the other hand if pteroylglutamic acid is given by mouth or by injection in doses of 4 or 5 mg., the pernicious anemia patient and the sprue patient excretes less than does the control. This may, however, be due to a difference in glomerular filtration rate since the controls used have been normal persons and not patients suffering from other forms of anemia of comparable degree. In normal persons, 0.1 per cent of a larger test dose of pteroylglutamic acid has been found to be excreted as citrovorum factor,⁹⁵ but there is a three fold increase of the excretion of the latter when ascorbic acid is given with the folic acid.⁹⁶ The urine of pernicious anemia patients and controls contains traces of citrovorum factor, and when the
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synthetic form of the latter was given intramuscularly, part of it was rapidly excreted.45

There is some evidence to suggest that vitamin B12 may be present in the urine in small quantities even in pernicious anemia.79, 45 Vitamin B12 given parenterally to pernicious anemia patients is partly rapidly excreted16, 97, 98 in a microbiologically active form, while Ross55 has produced evidence to suggest that it circulates for a short time in a microbiologically active form and then in a microbiologically inactive form for several weeks. It may be released from this inactive form by heating the diluted serum at 100 C. for a half to one hour. If this work is confirmed, it suggests that vitamin B12 is changed to a microbio-

logically inactive form, or combined with something other than Castle's intrinsic factor, or that in pernicious anemia there is a circulating thermolabile inhibitor for the growth of the test organism. Heated normal sera also supported the growth of the test organism which in this case was Euglena.

It has been shown that even in normal subjects, very large amounts of vitamin B12 (9 mg.) given by mouth lead to the excretion of only small quantities (1 \(\mu\)g.) in the urine,97, 98 and suggested97 that this may indicate poor absorption of vitamin B12 or alteration of vitamin B12 in some way during its passage through the gastro-intestinal tract so that it can no longer be excreted by the kidneys. The third alternative suggested is that B12 may undergo some change during its absorption that renders it ineffective in supporting the growth of the test organism. It would be of interest to explore this third possibility by examining the serum using Ross’s technic.

These three alternatives are not mutually exclusive and it is possible that the change to a microbiologically inactive form may occur after the vitamin has passed through the intestinal wall.43 Little work has been done on the vitamin B12, folic acid or citrovorum factor content of human tissues, but no vitamin B12 was found in the tissues of a patient who died of untreated pernicious anemia.45 In normal human autopsy liver, growth activity for S. fecalis was entirely due to folic acid. In a patient with pernicious anemia in relapse dying the day after receiving 100 \(\mu\)g. of vitamin B12, much of the activity was due to folic acid.59 Swendseid et al.100 have shown mouse liver to contain folicin but not folic acid, and have found that vitamin B12 is concentrated in the mitochondria, whereas folicin acid is more generally distributed throughout the cell. As far as could be ascertained by microbiologic assay there was evidence for the presence of vitamin B12 in skin biopsy specimens of 2 pernicious anemia patients in relapse.99

These are some of the findings that have to be considered in any theory about the antianemic factors that is based on investigations in the human.

Soon after the synthesis of pteroylglutamic acid was announced, it was suggested by several workers101, 102 that the basic defect in pernicious anemia is a failure of the liberation of pteroylglutamic acid from its conjugates, a step presumably normally carried out by vitamin B12. This was largely based on the finding that when pteroylhexaglutamylglutamic acid was administered to normal individuals there was an increased urinary excretion of pteroylglutamic acid, whereas this was not so in pernicious anemia patients in whom, too, there was no hematologic response. It was subsequently found, however, that certain yeast concentrates which served as sources of the natural folic acid conjugate contained
"conjugase inhibitors" which partly accounted for the diminished output of pteroylglutamic acid in pernicious anemia patients given pteroylhexaglutamylglutamic acid. Nevertheless, such patients did appear to lack the ability to utilize conjugates normally, and perhaps were more susceptible to the action of conjugase inhibitors. The further application of this work has not been found possible because of the difficulty of obtaining the natural conjugate of folic acid without the inhibitors.

Nevertheless, there has been a considerable amount of support for the theory that vitamin B₁₂ functions by releasing pteroylglutamic acid from its conjugate form or by making it available in some other way; this implies that pteroylglutamic acid is the substance that maintains normoblastic blood formation. However, recent work on the citrovorum factor would suggest that a further step is the conversion of pteroylglutamic acid to citrovorum factor. The latter is more effective than pteroylglutamic acid in reversing aminopterin inhibition in pernicious anemia.³

There are, however, serious objections to this theory which now derives most of its support from the lack of satisfactorily documented reports of cases of megaloblastic anemia that have failed to respond in the first instance to pteroylglutamic acid. The author has seen one patient with pernicious anemia who did not respond to 30 mg. of pteroylglutamic acid, but this in itself is not a very serious objection. Numerous authors have shown that patients with megaloblastic anemia including some with pernicious anemia will respond hematologically to the synthetic conjugates pteroyldiglutamic acid and pteroyltriglutamic acid,¹⁴,¹⁰⁸⁻¹⁰⁹ and that the pernicious anemia patient can excrete these forms in the urine as pteroylglutamic acid. This in itself is not sufficient justification for the conclusion of Wilkinson and Israels¹⁰⁷ that neither folic acid nor its conjugate play any major part in the etiology of the human pernicious anemia syndrome, since what happens with the synthetic conjugates does not necessarily bear any relation to what happens with the natural form pteroylhexaglutamylglutamic acid, and moreover, it has been shown that folic acid antagonists will prevent the response of pernicious anemia patients to vitamin B₁₂.¹⁰⁸,¹⁰⁹ We have already seen that there is definite evidence that the interrelationship between the various hemopoietic factors in bacterial and animal metabolism is more complex than this theory would suggest it to be in the human. In addition, however, there is the clinical objection that it has now been shown¹¹⁰⁻¹¹² that hematologic relapse will frequently occur in patients with pernicious anemia treated for periods of a year or more with pteroylglutamic acid. The patients will then respond to liver or vitamin B₁₂ therapy.

By the kindliness of Dr. W. A. Alexander of Edinburgh Royal Infirmary it has been possible to carry out investigations on a patient, age 23, suffering from the sprue syndrome, who had a very markedly megaloblastic marrow despite the fact that he had been taking pteroylglutamic acid by mouth in a dosage of 5 mg. daily for more than two years with hematologic improvement at the commencement of treatment. A test dose of 5 mg. of pteroylglutamic acid given by mouth on the present admission resulted in a urinary excretion of only .015 mg., which, in the light of present knowledge might suggest malabsorption of folic acid in the unconjugated form. There was no hematologic response whatsoever to 20 mg. pteroylglutamic acid daily by mouth for 10 days. A test dose of 5 mg. by injection then gave the low urinary excretion of 0.85 mg. suggesting either tissue depletion of folic
acid or conversion of much of the folic acid to some substance that did not support the growth of S. fecalis or alternatively was not excreted by the kidneys. There was no hematologic response to 15 mg. of pteroylglutamic acid daily intramuscularly for 7 days, the marrow remaining frankly megaloblastic; 90 mg. daily given intramuscularly for 14 days converted the marrow and led to a rise in red cells. Thereafter 90 mg. intramuscularly was given for five months. The red cell rise was not maintained, and the marrow reverted to the megaloblastic state.

Conclusions

This review of a vast literature is necessarily incomplete, and it is possible that investigations that may eventually prove to be of great importance have been omitted. The work reviewed here indicates that vitamin B$_12$, pteroylglutamic acid and the citrovorum factor are probably all concerned with the metabolism of the maturing red cell, quite apart from their other undoubted metabolic functions which have not been considered. Pteroylglutamic acid and vitamin B$_12$ play a part in methyl metabolism and in the metabolism of glycine, serine, ethanolamine and formic acid, all of which are probably concerned both with the formation of purines and pyrimidines and also with the synthesis of porphyrins. This, too, may be significant in relation to blood metabolism.

Bone marrow cultures have suggested that substances may exist in the serum that inhibit normal blood formation. If this is true of what takes place in the body, we do not know the mode of formation of such inhibitors, or whether any part of the action of the antimegaloblastic factors is concerned with the overcoming of such inhibitors.

Two main theories about the interrelationships of these factors have been considered. The view that vitamin B$_12$ is concerned with the release of pteroylglutamic acid from its conjugates is unsatisfactory mainly because it does not explain why pernicious anemia patients cannot be maintained indefinitely in hematologic remission with synthetic pteroylglutamic acid. It must be admitted, however, that there still exists the possibility that one of the actions of vitamin B$_12$ is to do this.

The other theory, put forward in part by Wright, Skeggs and Huff$^{26}$ and elaborated by Vilter et al.$^{27}$ is based very largely upon bacteriologic findings and requires further investigation in relation to megaloblastic anemias in man. According to this theory, pteroylglutamic acid which may be temporarily effective in a dosage of as little as 1 mg. daily, acts by a "mass action" effect in pernicious anemia. Several workers have suggested that if it does act in such a way, traces of vitamin B$_12$ in the body may be used up in the process. There is some evidence$^{49}$ that vitamin B$_12$ may not entirely be lacking from the tissues in pernicious anemia. Subacute combined degeneration of the cord, the pathogenesis of which is uncertain, might then arise when vitamin B$_12$ depletion was complete or almost complete.$^{43}$

Finally, there is evidence for the presence of numerous forms of vitamin B$_12$ in the body, and to the complexities of the interrelationships of the various factors there is added the complexities of the metabolic interrelationships of the various forms of vitamin B$_12$.

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102 Girdwood, R. H.: Unpublished observations.
Analytical Review: The Interrelationships of Factors that Influence the Megaloblastic Anemias

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