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The Relationships between Vitamin B$_{12}$, Folic Acid and Folinic Acid

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The interrelationships between the various substances used for the treatment of the megaloblastic anaemias are complex and little understood. The vast amount of experimental work carried out in recent years has given us so many papers that it is almost impossible to keep track of them, but the jig-saw is as yet incomplete. In the short time available it is not possible to deal fully even with one aspect of the problems, which have been considered in more detail in a recent review (Girdwood, 1952a); to-day I shall refer briefly to some of the reported work and add data derived from a few metabolic studies carried out on patients.

Chemical interrelationships

Dr Lester Smith has discussed the formula of vitamin B$_{12}$ and related forms (Smith, 1952). As is known, the name folic acid is usually given to synthetic pteroylglutamic acid, although it is not certain that the two are identical (Robinson, 1951). Pteroylglutamic acid is not related chemically to vitamin B$_{12}$. It has long been considered that the chief form of folic acid present in foodstuffs is that found in yeast, pteroylhexaglutamylglutamic acid, a γ-linked peptide which has not been synthesized. Satisfactory experiments cannot be carried out with this substance because of the presence of 'conjugase inhibitors' in the yeast concentrates used as a source of this natural folic-acid conjugate.

Synthetic conjugates of pteroylglutamic acid are pteroyldiglutamic acid and pteroyltriglutamic acid (Diopterin and Teropterin, Lederle Laboratories Inc.). As far as is known, these do not occur in food and, in fact, the former does not occur naturally.

Folinic acid (Bond, Bardos, Sibley & Shive, 1949) appears to be the same substance as the citrovorum factor (Sauberlich & Baumann, 1948), so called because it will support the growth of the streptococcus *Leuconostoc citrovorum*. The chemical

structure of this substance is reported to be 5-formyl-5:6:7:8-tetrahydropteroyl-glutamic acid. We do not know much about conjugate forms of folinic acid but, as will be explained later, it is likely that in certain tissues and organs the activity that has been attributed to folic acid or its conjugates is due at least in part to folinic acid or its conjugates.

Microbiological interrelationships and quantitative estimation of these haemopoietic factors

Under suitable assay conditions, vitamin B_{12} is a growth factor for *Lactobacillus leichmannii*, *Lb. lactis* Dorner, *Euglena gracilis* and *Bacterium coli*.

I shall not consider further the difficulties involved in carrying out the assays, as this is dealt with by Ford (1952).
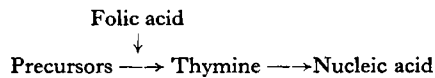
Pteroylglutamic acid is usually estimated by its growth-promoting effect upon *Streptococcus faecalis* R or *Lb. casei*. Pteroyldiglutamic acid will not support the growth of either of these organisms, whereas pteroyltriglutamic acid supports the growth of *Lb. casei* but not that of *Strep. faecalis* unless present in high concentration. Thymine and thymidine (thymine deoxyriboside) can replace pteroylglutamic acid for the growth of *Lb. casei* and *Strep. faecalis*.

The citrovorum factor is measured quantitatively by virtue of its growth-promoting effect upon *Leuc. citrovorum*. However, it will also support the growth of *Lb. casei* and *Strep. faecalis* and the true folic-acid content of substances being tested is arrived at by a differential assay method, using both *Lb. citrovorum* and *Strep. faecalis* as test organisms (Swendseid, Bethell & Ackermann, 1951). Under the conditions of my own differential assays, citrovorum factor has about 76 % of the growth-promoting effect of pteroylglutamic acid for *Strep. faecalis*.
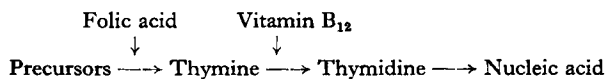
Thymidine can diminish the requirements of *Leuc. citrovorum* for folinic acid, but folic acid is inactive for *Leuc. citrovorum* unless present in very high concentration.

Other complications are many, and time does not permit me to mention them. I have found the folinic-acid assay to be far the most consistent and satisfactory. With the other assays difficulties are very frequent.
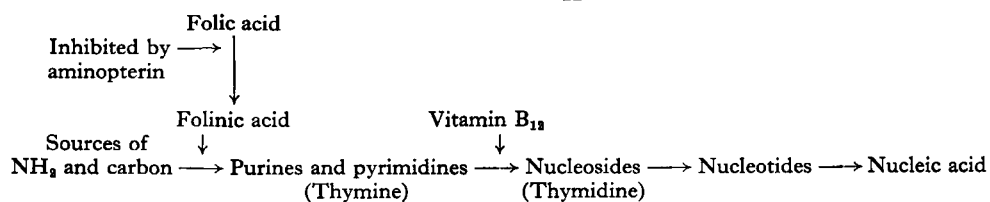
Stokes (1944) could not demonstrate folic acid in organisms grown in a medium containing thymine in place of folic acid and suggested that folic acid functions as a coenzyme for the enzyme system responsible for the synthesis of thymine, the latter being used by the bacteria to form nucleic acid:

$$\begin{array}{c} \text{Folic acid} \\ \downarrow \\ \text{Precursors} \longrightarrow \text{Thymine} \longrightarrow \text{Nucleic acid} \end{array}$$

Wright, Skeggs & Huff (1948) suggested that vitamin B_{12} functions as a coenzyme for the conversion of thymine to thymidine:

$$\begin{array}{c} \text{Folic acid} \qquad \text{Vitamin } B_{12} \\ \downarrow \qquad\qquad \downarrow \\ \text{Precursors} \longrightarrow \text{Thymine} \longrightarrow \text{Thymidine} \longrightarrow \text{Nucleic acid} \end{array}$$

Nichol & Welch (1950) produced evidence to support the view that folinic acid is the 'biologically active' form of folic acid and hence we have one theory of the interrelationships between these factors as follows:

```
                        Folic acid
        Inhibited by ──→  │
        aminopterin       │
                          ↓
                  Folinic acid              Vitamin B₁₂
        Sources of      ↓                       ↓
        NH₂ and carbon ──→ Purines and pyrimidines ──→ Nucleosides ──→ Nucleotides ──→ Nucleic acid
                        (Thymine)              (Thymidine)
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This has been elaborated by Vilter, Horrigan, Mueller, Jarrold, Vilter, Hawkins & Seaman (1950), who claim that thymine is effective in large doses in pernicious anaemia even after folic acid has had no effect. Hausmann (1949) has reported a response to thymidine in large dosage in pernicious anaemia, but I have been unable to obtain sufficient of this substance to carry out clinical experiments.

Interrelationships in animal experiments

Megaloblastic anaemia can be produced in certain animals, notably pigs, by dietetic means, by the use of folic-acid antagonists, or by both. It is considered that by such means the animals may be made deficient in both vitamin B$_{12}$ and folic acid. Responses have occurred following treatment with pteroylglutamic acid, pteroyldiglutamic acid, pteroylhexaglutamylglutamic acid (Heinle, Welch, George, Epstein & Pritchard, 1947, Cartwright; Tatting, Ashenbrucker & Wintrobe, 1949). Responses to liver injections, vitamin B$_{12}$, proteolysed liver, marmite, deoxyribose-nucleic acid, ribosenucleic acid, thymine and histidine have been poor (Cartwright, Palmer, Tatting, Ashenbrucker & Wintrobe, 1950). There is one paper describing pigs that appeared to become so depleted of vitamin B$_{12}$ and folic acid that folic acid failed to give a response until vitamin B$_{12}$ injections (liver injections) were given and, conversely, liver injections failed unless folic acid was given (Heinle, Welch & Pritchard, 1948). In general, the experiments indicate the importance of supplying both vitamin B$_{12}$ and folic acid to prevent megaloblastic anaemia in the pig. Protein deficiency increases the need for folic acid.

In considering megaloblastic anaemia in the monkey, we are faced with the problem of Wills's factor. As is well known, Wills studied nutritional megaloblastic anaemia in man in India, and in monkeys (Wills & Stewart, 1935; Wills, Clutterbuck & Evans, 1937; Wills & Evans, 1938). Anahaemin (British Drug Houses Ltd.), which was active in pernicious anaemia, was inactive in nutritional megaloblastic anaemia. Campolon (Bayer Products Ltd.) was active in pernicious anaemia and, when separation with ammonium sulphate was carried out, both the soluble and insoluble fractions were active in this disorder. However, only the soluble fraction was active in nutritional megaloblastic anaemia in man and in the monkey. Strauss & Castle (1932) state that the autolysed yeast preparation, marmite, which does not contain vitamin B$_{12}$, is active by mouth in pernicious anaemia only if given with gastric juice. However, marmite was active in nutritional megaloblastic anaemia. The main problem is whether Wills's factor is folic acid, folinic acid, or some other unindentified substance. The monkeys received 5 g of marmite daily (Wills & Stewart, 1935) an amount that is said to contain 0·3 mg of growth factors for *Strep. faecalis* (as pteroyl-glutamic acid). However, Day & Totter (1948) have shown that this is more than sufficient to protect the animals against anaemia. The normal daily intake of folic or

folinic acid required to prevent nutritional megaloblastic anaemia in man is uncertain; it is probably less than 100 μg/day; the minimal amount required to treat a patient with pernicious anaemia is quite another matter. It is known that the content of folic and folinic acid in present-day liver extracts is very small (usually less than 1·5 μg/ml.), but what we really would need to know is the content of these factors in Campolon in 1931–7 when Wills was doing her researches, and attempts are being made at present to obtain such material. The action of marmite in Wills's experiments could be explained on its content of growth factors for *Strep. faecalis*, though why marmite is not more effective in pernicious anaemia or why gastric juice potentiates its action is not fully understood. Perhaps the folic or folinic acid of marmite is present in a conjugate form from which it is released or made available by gastric juice, or perhaps gastric juice neutralizes inhibitors. Full understanding of the significance of separation by ammonium sulphate would appear to necessitate a knowledge of the effect of such separation not only upon folic acid and folinic acid but also upon their conjugates.

To complicate the experiments with monkeys further, it is now known that in the monkey placed on a deficient diet, megaloblastic anaemia develops more rapidly if there is ascorbic-acid deficiency. When it does develop, folic acid is rapidly effective in treatment, ascorbic acid is more slowly effective, vitamin B_{12} is ineffective, and vitamin B_{12} together with ascorbic acid gives a prompt response (May, Nelson, Lowe & Salmon, 1950). In infants, too, megaloblastic anaemia due to folic-acid deficiency occurs more rapidly if there is ascorbic-acid deficiency.

I shall not deal at all with the animal protein factor or with the effects of antibiotics, as these are being discussed separately (Coates, 1952; Cuthbertson, 1952).

Other metabolic interrelationships

It is now clear that folic acid and vitamin B_{12} take part in many processes concerned with the utilization of proteins not only for haemopoiesis but also for growth and development. For example, it has been shown in chicks that folic acid is involved in the conversion of choline to betaine and in transmethylation from betaine to methionine (Dinning, Keith & Day, 1951). Vitamin B_{12} also functions in methionine formation (Oginsky, 1950). Again, glycine toxicity in young chicks can be overcome by vitamin B_{12} and to a lesser extent by folic acid, but the two together are more effective than either alone (Machlin, Denton & Bird, 1951). These and other interrelationships could well be the subject of a symposium in themselves.

Bone-marrow cultures

In bone-marrow cultures, Rusznyák, Löwinger & Lajtha (1947) showed that the serum contained a factor that inhibited the conversion of megaloblasts to normoblasts. Folic acid and folinic acid caused such conversion, whereas vitamin B_{12} was ineffective except when mixed with a source of intrinsic factor (Callender & Lajtha, 1951). Lajtha (1950) has suggested that folic acid (or folinic acid) acts directly on the red-cell precursors, but that this activity is inhibited normally by a factor in the serum. This inhibitory factor is counteracted by vitamin B_{12} after the latter has been converted to an active form in the body. On the other hand, Horrigan, Jarrold & Vilter (1951),

who instilled folic acid, folinic acid and vitamin B$_{12}$ directly into the bone marrow of patients, reported that, whereas vitamin B$_{12}$ would convert megaloblastic cells to the normoblastic state, folic acid and folinic acid were not effective. This last approach requires further study.

Metabolic and therapeutic studies in man

At an early stage in the investigations of the action of folic acid in the megaloblastic anaemias, Bethell, Meyers, Andrews, Swendseid, Bird & Brown (1947) suggested that an important function of the liver factor (vitamin B$_{12}$) is to liberate folic acid from its conjugates, and that this cannot occur in pernicious anaemia. This conclusion was based upon measurements of urinary excretion of folic acid in pernicious-anaemia patients and controls after the administration of the naturally occurring conjugate, pteroylhexaglutamylglutamic acid. Unfortunately difficulties arose in confirming this work because it was found that the sources of the natural conjugate contained 'conjugase inhibitors' that complicated the experiments without altogether invalidating them. In support of this theory was the fact that all forms of megaloblastic anaemia responded in the first instance to folic-acid therapy. It is now known that in some patients treated for a period of years with folic acid, haematological relapse may occur. I should like to describe briefly such a patient, part of whose story has already been published (Girdwood, 1952a).

The patient was a male aged 23 years, suffering from the sprue syndrome with 68 % absorption of fat; the test meal showed histamine-fast achlorhydria. He had a markedly megaloblastic marrow although he had been taking folic acid by mouth in a dosage of 5 mg daily for more than 2 years. As will be seen from Fig. 1 there was then no response to 15 mg folic acid daily intramuscularly, but 90 mg daily converted the marrow to the normoblastic state and led to a rise in the red cells. This improvement was not maintained with 90 mg of folic acid intramuscularly weekly, and the patient was readmitted to hospital in November 1951, with pneumonia. The marrow was again megaloblastic.

The lung infection did not respond well to sulphonamide therapy. I was anxious to try the effect of vitamin B$_{12}$ on the blood picture of this patient who was not in the ward to which I am attached. Unfortunately on the very day that this treatment was given the doctor who was responsible for attending to the patient was himself taken ill and penicillin was given to the sprue patient without my prior knowledge. This perhaps complicates matters since there have been reports from Africa of responses to penicillin in megaloblastic anaemia, but in any event it can be said that the blood picture was restored rapidly to normal although the haemopoietic tissues had become refractory to very large doses of folic acid.

I imagine that the most likely explanation of this response is that the patient was unable to absorb vitamin B$_{12}$ and that folic acid could not be effective in its absence. It might be suggested that folinic acid would have been effective and that perhaps the function of vitamin B$_{12}$ is to play some part in the conversion of folic acid to folinic acid. However, Jarrold, Horrigan, Thompson & Vilter (1951) have reported on a patient with pernicious anaemia who became refractory to folic acid. There was no response to Lederle's synthetic preparation of citrovorum factor, but vitamin B$_{12}$ was effective.

In support of the theory that vitamin B_{12} releases folic acid from its conjugates, it has been reported (Suárez, Welch, Heinle, Suárez & Nelson, 1946; Anonymous, 1947) that liver-extract injections give rise to increased urinary excretion of folic acid in pernicious anaemia and sprue. As will be seen in Fig. 2, that has not been our experience.
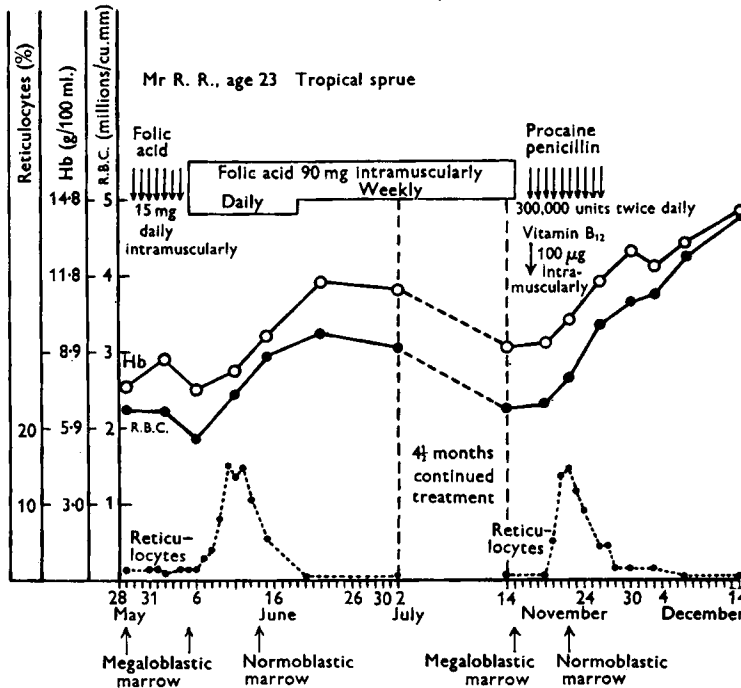

Fig. 1. Megaloblastic anaemia refractory to folic-acid therapy.

If synthetic folic-acid conjugates are given by mouth or by injection to patients with pernicious anaemia, there is a haemopoietic response and excretion of pteroylglutamic acid. Table 1 shows the urinary excretion of pteroylglutamic acid in eight patients treated in this way—in only two patients given the dose indicated for several days was there no haemopoietic response. All that this experiment shows, however, is that the pernicious-anaemia patient can convert these particular synthetic forms to 'free' folic acid. For comparison, the average excretion of folic acid in the urine of eight pernicious-anaemia patients given 5 mg pteroylglutamic acid intramuscularly was 1·05 mg.

Finally, I want to refer briefly to a few of the metabolic mysteries of the megaloblastic anaemias. Mollin & Ross (1951), using *Euglena gracilis* as test organism, have found that the urinary and serum values for vitamin B_{12} are lower in pernicious-anaemia patients than in controls. On the other hand, using *Lb. leichmannii* (and correcting for deoxyribosides) we have found similar amounts of vitamin B_{12} in the urine in pernicious-anaemia patients and in normal persons. One possibility is that the *Lb. leichmanni* assay estimates as yet unidentified substances other than vitamin B_{12}
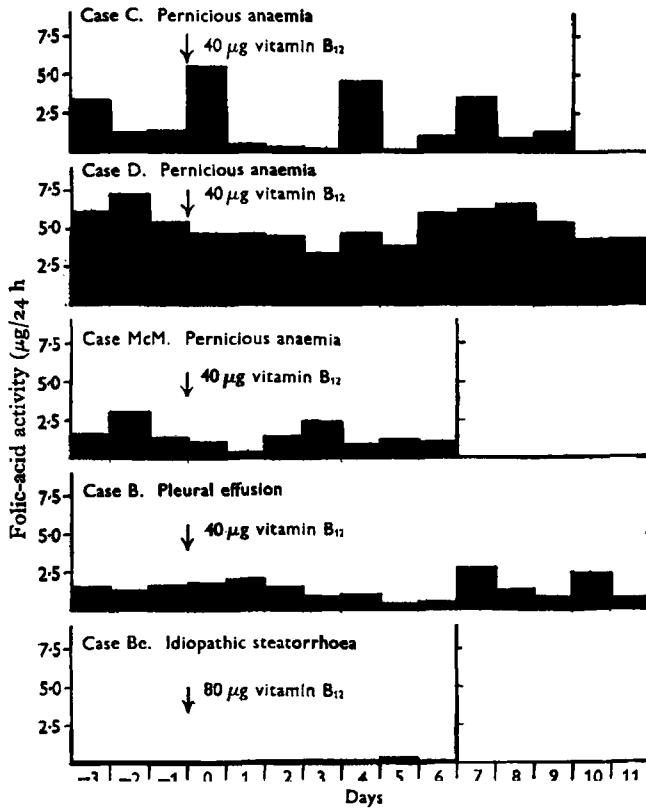
Fig. 2. Effect of vitamin B$_{12}$ given intramuscularly on total folic-acid activity of urine.

Table 1. *Urinary output of pteroylglutamic acid following administration of its synthetic conjugates in pernicious anaemia*

| Case | Drug | Dose (mg) | Route* | Folic-acid activity of urine | | Remarks |
				Before treatment (μg/24 h)	After treatment (mg/24h)	
McL.	Pteroyltriglutamic acid	10	IM	1·6	1·880	—
C.	Pteroyltriglutamic acid	10	Oral	2·8	1·440	—
R.	Pteroyltriglutamic acid	2·5	Oral	3·2	0·036	—
McG.	Pteroyltriglutamic acid	2·5	Oral	1·5	0·006	—
P.	Pteroyldiglutamic acid	5	IM	1·1	0·280	No haematological response with 18 days' treatment. Response to 5 mg PGA, IM, daily
L.	Pteroyldiglutamic acid	5	Oral	1·3	0·330	—
H.	Pteroyldiglutamic acid	2·5	IM	0·3	0·370	No response
S.	Pteroyldiglutamic acid	2·5	IM	1·6	0·012	—

* IM = intramuscular.

that are destroyed by alkaline hydrolysis. If so, it is surprising that there is not a greater difference between the values for patients and controls. Recent results for the urinary content of haemopoietic factors are given in Table 2.

Table 2. *Urinary content of vitamin B_{12}, folic acid and folinic acid (citrovorum factor)*

Case	Diagnosis	Vitamin B_{12} content (mμg/24 h)	Folic-acid activity (μg/24 h)	Folinic-acid content (μg/24 h)	True folic-acid content (μg/24 h)
A.	Megaloblastic anaemia of pregnancy	Nil	1·60	1·92	0·14
G.	Pernicious anaemia	121·5	2·30	0·80	1·69
S.	Pernicious anaemia	108·1	1·61	0·45	1·27
H.	Pernicious anaemia	102·0	0·33	0·20	0·18
Ar.	Pernicious anaemia	71·7	2·90	0·41	2·59
Gi.	Pernicious anaemia	45·5	8·70	2·90	6·50
B.	Pernicious anaemia	36·5	0·97	0·34	0·71
W.	Pernicious anaemia	35·0	1·80	1·00	1·04
R.	Pernicious anaemia	32·4	2·12	0·92	1·42
S.	Pernicious anaemia	21·4	2·20	0·62	1·73
P.	Pernicious anaemia	Nil	2·40	0·73	1·84
T.	Pernicious anaemia	Nil	2·20	1·35	1·15
M.	Normal	198·6	2·03	0·52	1·64
Ro.	Normal	120·5	4·47	1·12	3·62
D.	Normal	Negligible	0·61	0·45	0·27
K.	Normal	Nil	3·98	0·76	3·47

Table 3. *Urinary excretion of citrovorum factor following its administration by mouth or by injection*

Case	Diagnosis	Treatment with Leucovorin* Dose (mg)	Route‡	Urinary excretion in 24 h after treatment Folic-acid activity† (*Strep. faecalis* assay) (μg/24 h)	Citrovorum-factor activity *Strep. faecalis* assay (μg/24 h)	*Lb. citrovorum* assay (μg/24 h)
F.	Pernicious anaemia	3	Oral	7·4	—	0·199
S.	Pernicious anaemia	3	Oral	18·7	30·8	30·4
		6	Oral	26·8	31·0	41·5
G.	Pernicious anaemia	12	Oral	155·0	103·3	135·9
Si.	Pernicious anaemia	12	Oral	28·7	39·7	30·6
Sn.	Pernicious anaemia	3	IM	202·0	—	302·0
W.	Pernicious anaemia	9	IM	1100·2	—	1560·4
C.	●Pernicious anaemia	12	IM	1850·5	—	1870·3
Sh.	Pernicious anaemia	12	IM	583·5	—	791·5
McF.	Pernicious anaemia	12	IM	352·4	—	480·2
A.	Megaloblastic anaemia of pregnancy	12	Oral	99·5	120·3	100·1
W.	Normal	12	Oral	99·5	134·8	70·6
McL.	Normal	12	Oral	99·6	133·6	76·7
Ro.	Normal	12	IM	1227·2	1761·0	1827·4
Re	Normal	12	IM	957·3	1264·4	1110·0

* Lederle Laboratories Inc.   † Due largely to citrovorum factor.   ‡ IM = intramuscular.

Pernicious-anaemia patients will respond to 3–12 mg of folinic acid whether it be given by mouth or by injection, the smaller doses being administered daily (Davidson & Girdwood, 1951 *a, b*). However, as will be seen from Table 3, the urinary output

of folinic acid is much less when it is given by mouth, although it is haemopoietically active by both routes and is not excreted as folic acid. If this is merely a matter of poor absorption by the alimentary route, it is surprising that oral treatment is satisfactory. In contrast, if folic acid is given in comparable dosage, the urinary output of folic acid is similar whether it be given by injection or by mouth, and only a small proportion (0·1 % or less) is excreted as the citrovorum factor.

The livers of two non-anaemic control patients examined at autopsy contained 162·3 and 523·7 $\mu g/100$ g respectively of citrovorum factor, but no folic acid. At autopsy, the liver of a pernicious-anaemia patient who died the day after having been given 100 μg. vitamin B_{12}, contained 78·9 μg citrovorum factor and 61·6 μg folic acid per 100 g (Girdwood, 1952b). This should only be taken as an indication for further investigation along similar lines.

Holly (1951), acting on the suggestion of May *et al.* (1950) that ascorbic-acid deficiency may interfere with the conversion of folic acid to folinic acid, has treated patients with megaloblastic anaemia of pregnancy with vitamin B_{12} and ascorbic acid. In three such patients, vitamin B_{12} and ascorbic acid separately were ineffective, whereas the two together gave a haematological remission.

These results, if confirmed, suggest that, as in the megaloblastic anaemias of infants and of monkeys, ascorbic-acid deficiency plays a part in the causation of at least some cases of megaloblastic anaemia of pregnancy.

Conclusions

All that we can conclude at present is that vitamin B_{12} and folic acid both appear to be required for normoblastic blood formation. It is probable that folic acid is normally converted in the body to folinic acid, but this latter substance is not the end of the metabolic chain. There is some indirect evidence for the presence of inhibitory factors (Lajtha, 1950; Ungley, 1951), but their existence is still problematical. Ascorbic-acid deficiency appears to be related to the development of megaloblastic anaemias, although this form of anaemia is not found in scurvy itself. The existence of other factors is possible. We have seen that Wills's factor has not yet been satis-factorily explained. Moreover, in certain forms of anaemia where the marrow is frequently megaloblastic (e.g. in sprue) there are some cases where the marrow is not truly megaloblastic but contains cells intermediate between megaloblasts and normo-blasts. This type of anaemia frequently fails to respond to vitamin B_{12} or folic acid, possibly because some other unknown factor is lacking.

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The Microbiological Assay of Vitamin B_{12}

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The literature on the microbiological assay of vitamin B_{12} is large and growing. Those familiar with microbiological techniques realize their limitations, but there is a danger that the apparent ease with which the tests can be carried out may encourage uncritical acceptance of the spate of results now appearing. An object of this paper is to show some of the difficulties that may be involved in the assay of vitamin B_{12}.

To begin with there are the ever-present fundamental problems of specificity of test and completeness of extraction. There is next the problem of the total extraction and measurement as vitamin B_{12} of the series of related substances, all active microbiologically, that may be present in natural products, and that are distinguished from vitamin B_{12} only by the replacement of the cyano-group by a variety of complex-bound anions (Brink, Kuehl & Folkers, 1950; Kaczka, Wolf, Kuehl & Folkers, 1950; Smith, 1948; Smith, Fantes, Ball, Ireland, Waller, Emery, Anslow & Walker, 1951; Pierce, Page, Stokstad & Jukes, 1949; Wijmenga, Veer & Lens, 1950).

A further problem arises from the presence in some natural materials of other