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Vitamin B_{12} absorption in megaloblastic anaemia

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1. The response to cyanocobalamin and folic acid therapy was studied in forty-seven subjects with severe nutritional megaloblastic anaemia; twenty-five were vitamin B_{12} deficient and, of these, ten were also folic acid deficient.

2. In twenty-four of the subjects, absorption of orally administered cyanocobalamin was normal, and there was a haematological response to small doses of orally administered cyanocobalamin, indicating adequate absorption. In one subject there was impaired cyanocobalamin absorption although free gastric hydrochloric acid was present.

3. The findings indicate that the absorptive function of the small intestine was unimpaired, with one exception, although there was a possible defect in nucleoprotein synthesis associated with a lack of vitamin B_{12} and folic acid. Addisonian pernicious anaemia was not found in any of the subjects.

Vitamin B_{12} and folic acid coenzymes play an essential part in the synthesis of nucleoprotein in mammalian cells. In their absence there is a defective rate of nucleoprotein synthesis which, although best documented in cells of the bone marrow, probably occurs in other cells. Morphologically abnormal cells have been found in the oral and gastric epithelium, in the upper gastrointestinal and respiratory tracts and in the mucosa of the small intestine of subjects with pernicious anaemia (Foroozan & Trier, 1967). The morphological abnormalities found in the mucosa of the stomach and small intestine are of particular interest, as it has been shown that in man, intrinsic factor is secreted by the parietal cells of the stomach, and that the vitamin B_{12}–intrinsic factor complex is absorbed by the microvilli of the epithelium of the distal ileum (Booth & Mollin, 1959). In severe vitamin B_{12} or folic acid deficiency it is possible that either or both of these mechanisms is damaged, resulting in malabsorption of the haematinic agent when given orally. Malabsorption of orally administered vitamin B_{12} associated with a defect in the ileum has been found in pernicious anaemia (Haurani, Sherwood & Goldstein, 1964; Carmel & Herbert, 1967). However, in this disorder in addition to the possible effects of the lack of vitamin B_{12} there is the complicating factor of autoimmunity with possible damage to the intestinal mucosa on an autoimmune basis. The effects of simple vitamin B_{12} deficiency would be better studied in subjects whose diet is deficient in vitamin B_{12}, without the complication of autoimmunity. Information on the absorption of vitamin B_{12} in this type of subject is very limited, mainly because of their rarity in the Western world.

Schloesser & Schilling (1963) reported studies on a single Asiatic Indian vegetarian with megaloblastic anaemia which was not responsive to orally administered vitamin B_{12} although sufficient quantities of intrinsic factor were present. They postulated

that there was a functional failure of the vitamin B_{12} absorbing mechanism in the ileum caused by a deficiency of the vitamin. If this finding is confirmed it would be of considerable importance in countries in which a dietary deficiency of the vitamin rather than an absence of intrinsic factor, is the commonest cause of a deficiency. It would mean that these subjects could not be successfully treated by administration of oral vitamin B_{12} (either as a therapeutic dose or by increasing the dietary intake of the vitamin) until their intestinal mucosa returned to normal. Since the conclusions were based on a study of a single subject, a larger study seemed desirable.

The present study was undertaken to determine whether there was a defect in absorption of the orally administered vitamin in subjects with severe vitamin B_{12} deficiency in Sri Lanka.

MATERIALS AND METHODS

Subjects and treatment. Forty-eight subjects with megaloblastic anaemia, diagnosed on bone marrow examination, were studied. All but two subjects were females, five were aged between 10 and 19 years, twenty-six were 20–29 years, nine were 30–39 years and six were 40–49 years. The two men were aged 14 and 30 years.

A peripheral blood film was studied and haemoglobin concentration, packed cell volume and fasting serum iron concentration were estimated. A bone marrow aspirate was examined for megaloblasts and stained for Fe. Subjects who had a critically low haemoglobin concentration (below 20 g/l) were transfused after the initial investigations had been done, and before they entered the trial.

The subjects were given the ward diet; reticulocyte counts were done daily and the haemoglobin concentration was estimated on alternate days. If there was no increase in the reticulocyte count over a period of 1 week, and if the initial investigations did not show evidence of Fe deficiency, the subject entered the trial. If there was a reticulocytosis or an increase in the haemoglobin concentration during the preliminary period, therapy was withheld until the haemoglobin level had stabilized. Those subjects who had evidence of an Fe deficiency were given an oral dose of ferrous sulphate (0·2 g three times daily) until the haemoglobin level had stabilized. If this was below the normal level and the bone marrow aspirate contained megaloblasts, Fe therapy was stopped and the subject then entered the trial.

Gastric juice was aspirated from the fasting subject, the subjects were given an intramuscular injection of 0·04 mg histamine/kg body-weight, and the aspiration repeated every 20 min for 1 h. The pH was measured and the hydrochloric acid content was determined by titration with 0·01 M-sodium hydroxide to pH 4·0.

The subjects were then given a daily oral dose of 4 μg cyanacobalamin, reticulocyte counts were done daily and haemoglobin concentration and packed cell volume were estimated on alternate days. If there was a reticulocytosis or increase in the haemoglobin concentration, therapy was continued until the haemoglobin levels showed no rise over a period of 10 d. If there was no response after 9 d of therapy, the cyanocobalamin dose was replaced by an intramuscular injection of 1 μg cyanocobalamin/d. If there was no response in the serial determinations of the reticulocyte count or haemoglobin concentration after 10 d, the intramuscular administration of cyanoco-

balamin was stopped, and the subjects were given 50 μg pteroylmonoglutamic acid/d. Those subjects in whom there was a response in the haemoglobin concentration when given cyanocobalamin orally (indicating adequate intestinal absorption) but whose haemoglobin concentration failed to reach normal levels, were not given parenteral cyanocobalamin. They, like those who failed to respond to parenteral cyanocobalamin, were given pteroylmonoglutamic acid orally. This vitamin was given to those subjects in whom vitamin B_{12} deficiency had already been excluded.

In three subjects, towards the end of the course of treatment with cyanocobalamin or pteroylmonoglutamic acid, the haemoglobin concentration, which had been showing a steady increase, showed no further increase and failed to reach normal levels. The serum Fe had fallen and it was assumed that a Fe deficiency had appeared, resulting from the rapid increase in erythropoiesis. When Fe was administered to these subjects, the haemoglobin concentration rose to normal levels. The haemoglobin concentration at the start of therapy varied from 30–40 g/l, and at the time Fe was given, varied from 89 to 110 g/l.

The cyanocobalamin for oral administration was prepared by diluting a 100 μg parenteral cyanocobalamin preparation in 100 ml distilled water and this solution was stored away from sunlight as cyanocobalamin is stable at room temperature if the pH is between 4 and 7, and the solution is kept away from bright light.

Analytical procedures. The haemoglobin was determined by the cyanmethaemoglobin method, and the packed cell volume estimated using a Hawksley microhaematocrit. Reticulocyte counts were done using brilliant cresyl blue. The serum Fe was determined by the dipyridyl method.

Faecal fat was determined by the method of van de Kamer, Huinink & Weyers (1949).

RESULTS

Response to cyanocobalamin and pteroylmonoglutamic acid

In one of the forty-eight subjects studied there was no response to small doses of cyanocobalamin and pteroylmonoglutamic acid. Later, she showed no response to massive doses of injected cyanocobalamin (100 μg/d), pteroylmonoglutamic acid (5 mg three times/d), ascorbic acid (100 mg/d) and protein supplements; blast cells appeared in the peripheral blood, and she was found to be a case of di Guglielmo's syndrome, where the marrow shows changes very similar to those seen in megaloblastic anaemia. This subject was excluded from the trial.

The response in the other forty-seven subjects is shown in Table 1. All the subjects responded to either cyanocobalamin or pteroylmonoglutamic acid, or both. Folic acid deficiency was commoner than vitamin B_{12} deficiency, although the difference in incidence between them was not statistically significant.

Megaloblastic anaemia due to vitamin C deficiency was not seen.

Response to cyanocobalamin

In fifteen subjects, cyanocobalamin alone was sufficient to restore the haemoglobin concentration to normal. The mean pretreatment haemoglobin concentration was

Table 1. *Response of forty-seven subjects with megaloblastic anaemia to treatment with cyanocobalamin and pteroylmonoglutamic acid (PGA)*

(Mean values with their standard errors)

Treatment	No. of subjects	Percentage response to treatment	Haemoglobin concentration (g/l)												Maximum reticulocyte count (% of red blood cell count)	Day at which maximum value was reached
			After cyanocobalamin given orally				After intramuscular injection of cyanocobalamin				After PGA given orally					
			Initial		Final		Initial		Final		Initial		Final			
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE		
Cyanocobalamin	14 1	31·9	38 43	3·0 —	126 42	1·8 —	42 42	— —	— 119	— —	— —	— —	— —	— —	7·0	12
Cyanocobalamin + PGA	10	21·3	41	2·9	86	6·2	—	—	—	—	86	6·2	127	2·7	7·0* 9·6†	5 8
PGA	22	46·8	53	3·1	51	3·6	51	3·6	49	3·8	49	3·8	129	2·1	11·0	7

* After cyanocobalamin.
† After PGA.

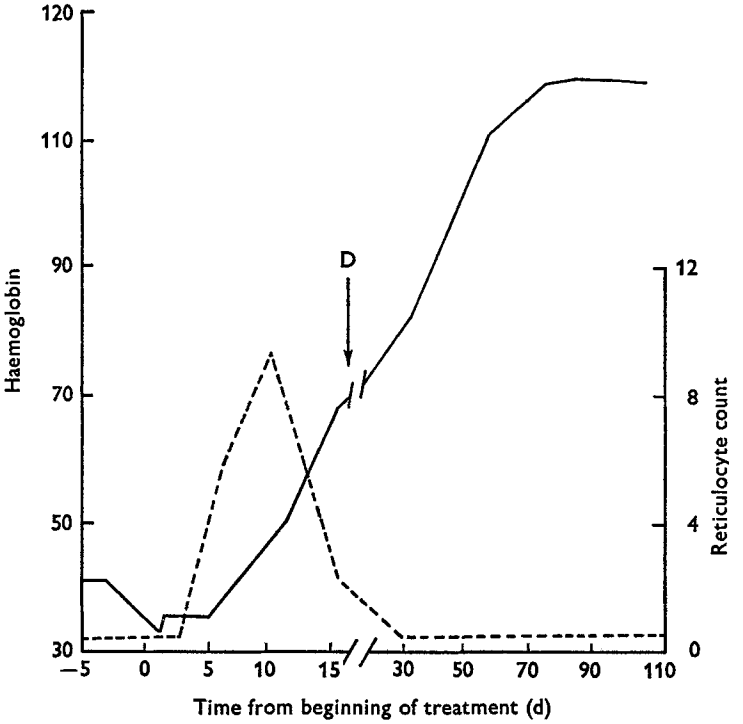

Fig. 1. A typical response in haemoglobin concentrations (g/l) (——) and reticulocyte count (% of total red blood cell count) (----), in a subject with simple vitamin B_{12} deficiency, to an oral dose of 4 μg cyanocobalamin/d. D, discharged from hospital.

38 g/l (range 21–69). In fourteen of these fifteen subjects, there was an immediate response to 4 μg cyanocobalamin/d given orally. A typical response is shown in Fig. 1. The initial and final haemoglobin concentration in this group and their reticulocyte response is shown in Table 1. The immediate response to a very low oral dose must indicate a normal secretion of intrinsic factor and a normal absorptive pathway in the small intestine.

In one of the fifteen subjects (Fig. 2) there was no response to orally administered cyanocobalamin (4 μg/d). After 9 d of therapy, there was no reticulocytosis, and the haemoglobin had fallen. Intramuscular administration of cyanocobalamin (1 μg/d) produced a rise in the reticulocyte count and haemoglobin concentration. The gastric juice showed a fasting pH of 5·2 which fell to 2·4 after an injection of histamine; the concentration of the free acid was 25 mequiv./l.

The effect of replacing cyanocobalamin by pteroylmonoglutamic acid

In ten subjects there was an increase in the haemoglobin concentration after oral administration of cyanocobalamin but the haemoglobin levels did not reach normal. The replacement of the cyanocobalamin dose by pteroylmonoglutamic acid produced an immediate increase in the haemoglobin concentration to reach normal levels. This finding indicates that although there was severe folic acid deficiency, there was no defect in the absorption of vitamin B_{12}.

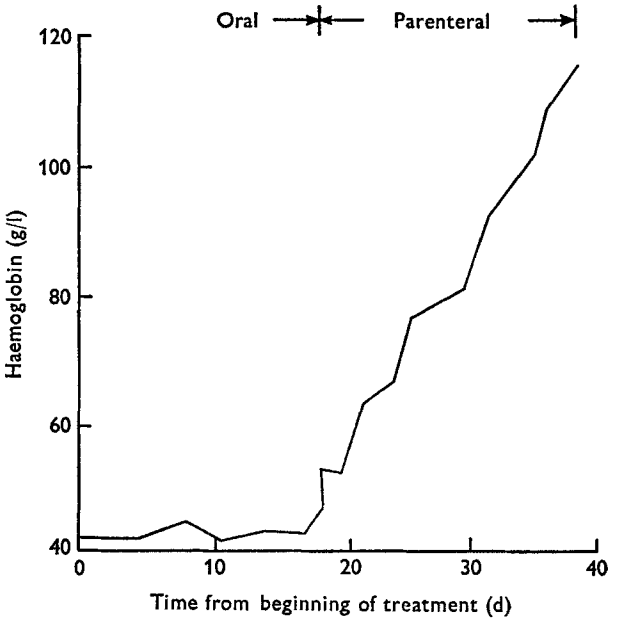

Fig. 2. Response in haemoglobin concentration to an intramuscular dose of 1 μg cyanocobalamin/d from day 10 in one subject who failed to respond to an oral dose of 4 μg cyanocobalamin/d for 9 d, although free gastric hydrochloric acid was detected.

Response to pteroylmonoglutamic acid

There were twenty-two subjects (46·8 %) in whom oral treatment with pteroylmonoglutamic acid resulted in a complete haematological remission with complete correction of the anaemia. The mean pretreatment haemoglobin concentration was 49 g/l (range 25–87).

Effect of the ward diet

The ward diet consisted mainly of rice with cooked vegetables and very little protein. In only three of the forty-seven subjects studied was there a response in either the haemoglobin concentration or the reticulocyte count in the week before treatment began. As the remaining forty-five subjects subsequently responded to oral doses of cyanocobalamin or pteroylmonoglutamic acid (or both), the diet supplied to our subjects must have contained less than 4 μg cyanocobalamin and 50 μg available folic acid/d, and was, therefore, unlikely to interfere with the effects of therapy.

Survey of subjects' home diet

In the group with simple vitamin B_{12} deficiency, only two of the fifteen subjects were absolute vegetarians. The other thirteen ate animal protein, although the total amount eaten was grossly inadequate.

In the folic acid-deficient group, all twenty-two subjects ate green vegetables. Vegetables were eaten cooked (as curries) and also as 'mallun', which is made from any chopped leafy vegetable lightly tossed in a warm pan. Cooking is minimal in a

'mallun' which is a common constituent of the diet here. All subjects ate a diet deficient in protein, and there was no difference between the diet of this group and that of the group with simple vitamin B_{12} deficiency.

Gastric acid

Free gastric hydrochloric acid was found in all forty-seven subjects. The average pH after injection of 0·04 mg histamine was 2·21 (range 1·2–3·5) and the average free acid concentration was 24·0 mequiv./l.

Malabsorption

There was no evidence of steatorrhoea in any of the subjects. The fat excretion was less than 4 g/24 h and the intake was 70 g/d (fat was added to the normal diet for 3 d before and during the collection of faeces).

DISCUSSION

Assessment of vitamin B_{12} deficiency

In this study, the time-honoured reticulocyte method of Minot & Castle (1935), and the changes in haemoglobin concentration after very small doses of cyanocobalamin or pteroylmonoglutamic acid, were used as an index of vitamin B_{12} or folic acid deficiency. We believe that the reticulocyte count should be combined with serial haemoglobin concentrations as the haemoglobin concentration sometimes increases without a preceding increase in the reticulocyte count. In many of our subjects there was a much quicker response in the haemoglobin concentration than is usually reported. This immediate response, often occurring within a few days of dosing with an effective haematinic, raises the question of the validity of the reticulocyte count with all its known inaccuracies. Further, in a mild haemolytic state, which is not uncommon in megaloblastic anaemia, the reticulocyte count is raised, and is therefore invalid as an index of a response to haematinics. We now use serial estimations of haemoglobin concentration rather than the reticulocyte response, as an index of effective therapy.

Several workers (Zalusky, Herbert & Castle, 1961; Hansen & Weinfeld, 1962) have established beyond doubt the diagnostic value of the administration of small doses of vitamin B_{12}. A response of megaloblastic anaemia to a daily dose of 1–5 μg cyanocobalamin virtually establishes a deficiency of this vitamin. Herbert (1963) suggests that in a clinical study involving vitamin B_{12} and folic acid treatment, vitamin B_{12} should be administered first. With a constant diet and adequate pretreatment investigations, a response in the reticulocyte count and haemoglobin concentration done serially is as reliable as information obtained from plasma concentration of these haematinic agents, and is less liable to errors of technique. Although the method is time-consuming, the information obtained is in no way inferior to that obtained by more elegant techniques. Streiff (1970), in a recent authoritative review of folic acid deficiency, states that the result of a clinical trial of oral doses of folic acid is still needed to diagnose folic acid-deficiency anaemia. Sullivan (1970) confirmed that there was agreement between the results obtained by measuring the plasma vitamin B_{12} concentration and

the conclusions drawn from a clinical trial with cyanocobalamin in vitamin B_{12}-deficient subjects.

The Schilling test (Schilling, 1953) is widely used as a test of vitamin B_{12} absorption. However, we think this test is unsatisfactory because of the confusion that would result from the administration of large doses of vitamin B_{12}. In both the original test described by Schilling, and its subsequent modifications, a large dose (1000 μg) of vitamin B_{12} is given as a 'flushing' dose. A reticulocytosis and an increase in the haemoglobin concentration could be produced in a simple folic acid deficiency by this very large dose of vitamin B_{12} as discussed later.

Cyanocobalamin absorption

In this study, twenty-five of forty-seven (53·2%) subjects with megaloblastic anaemia were shown to have vitamin B_{12} deficiency, either as simple vitamin B_{12} deficiency (fifteen subjects), or as combined vitamin B_{12} and folic acid deficiency (ten subjects). The deficiency was almost certainly of dietary origin (see later discussion) and free gastric hydrochloric acid was present in all the subjects. The subjects were severely anaemic, with an average haemoglobin concentration of 38 g/l (range 21–69. In all subjects, megaloblasts were present in the bone marrow, and in some, the megaloblastic changes were very severe. Although there was evidence of severe vitamin B_{12} deficiency, there was an immediate response to a small oral dose of cyanocobalamin (4 μg/d), indicating the presence of intrinsic factor and a functionally normal vitamin B_{12}–intrinsic factor absorption mechanism in the small intestine. It would therefore appear that, although the small intestinal epithelium proliferates rapidly and has a regeneration time of only 4–6 d (Lipkin, Sherlock & Bell, 1963), the possible defect in nucleoprotein synthesis associated with a lack of vitamin B_{12} does not affect the functional integrity of these cells. In only one of the twenty-five subjects (see later discussion) was there evidence to suggest a defect in the absorption of orally administered cyanocobalamin.

Intestinal malabsorption of cyanocobalamin is common in pernicious anaemia, even with oral administration of intrinsic factor (Haurani *et al.* 1964). This malabsorption has been interpreted as ileal dysfunction secondary to vitamin B_{12} deficiency (Carmel & Herbert, 1967). Our findings do not support this suggestion. The rarity of pernicious anaemia in Sri Lanka might explain the normal intestinal absorption of cyanocobalamin in our subjects. The present views on pernicious anaemia as an autoimmune disease raise the possibility of damage to the intestinal mucosa by an autoimmune mechanism. As this autoimmune damage is not present in vitamin B_{12} or folate deficiency of dietary origin, there is no intestinal malabsorption.

Our over-all findings do not support those of Schloesser & Schilling (1963). The study reported by these workers may be criticized as they failed to obtain a reticulocyte response to an oral dose of 1 μg cyanocobalamin but obtained a positive response to an injected dose of 1000 μg cyanocobalamin and concluded that there was a vitamin B_{12} deficiency. There is, however, serious doubt about the diagnostic significance of the haematological response to large doses of vitamin B_{12} or folic acid. Zalusky *et al.* (1961) showed that a reticulocyte response could be obtained in subjects with folic

acid deficiency who were given large doses of cyanocobalamin. If the reticulocyte response is to be used as a diagnostic test, the dose of cyanocobalamin given should be very small (1–4 μg/d). Under these circumstances, a reticulocyte response can be considered a reliable test of a pre-existing deficiency (Marshall & Jandl, 1960).

Therapy with antibiotics and corticosteroids could occasionally produce misleading results. Penicillin and tetracycline have been shown to produce a haematological response in subjects with megaloblastic anaemia (Foy, Kondi & Hargreaves, 1951), probably a result of their action on the intestinal flora (Foy & Kondi, 1954). Vitamin B_{12} absorption is said to improve in subjects with pernicious anaemia treated with corticosteroids. In our studies, none of the subjects was given antibiotic or corticosteroid treatment during the trial period.

In ten of the subjects, there was combined vitamin B_{12} and folic acid deficiency. However, there was intestinal absorption and a haematological response to an oral dose of 4 μg cyanocobalamin which does not support the finding (Hift & Adams, 1963; Scott, Kammer, Burger & Middleton, 1968) that folic acid deficiency may damage the intestinal mucosa sufficiently to result in malabsorption of vitamin B_{12}.

Cyanocobalamin malabsorption and gastric hydrochloric acid

One of our subjects failed to absorb orally administered cyanocobalamin although there was a normal acid output from the stomach (and presumably, an adequate supply of intrinsic factor). Although a selective failure of intrinsic factor secretion (in the presence of hydrochloric acid secretion) has been described (McIntyre, Sullivan, Jeffries & Silver, 1965) such examples are very rare. It is more likely that there was an adequate secretion of intrinsic factor but that the vitamin B_{12}–intrinsic factor complex could not be absorbed due to a functional failure of the ileal epithelial cells. Before this failure is accepted as a selective failure of cyanocobalamin absorption, a generalized malabsorption has to be excluded. Tropical sprue with ileal damage is a well-recognized cause of vitamin B_{12} malabsorption (Bayless, Wheby & Swanson, 1968), and this condition must always be considered before ascribing the malabsorption defect to a lack of vitamin B_{12} or folic acid. In the subject in our study, the faecal fat excretion was normal when the intake was 70 g fat/d, and the intestinal mucosa was morphologically normal on biopsy. These findings do not support a diagnosis of a generalized malabsorption.

A functional failure of the ileal epithelial cells could have been the result of a derangement of nucleoprotein synthesis associated with a lack of vitamin B_{12}. This mechanism was postulated by Schloesser & Schilling (1963) to explain similar findings in an Asiatic Indian vegetarian. A difficulty in accepting this hypothesis is that it is unlikely that such a functional defect of the ileal cells should be limited to the absorption of vitamin B_{12}.

We have resumed oral dosing of our subject with cyanocobalamin, and she is being observed in order to determine whether her vitamin B_{12} absorptive mechanism has returned to normal.

Diet

The diet of the subjects is of interest from two points of view. First, it is often stated that a dietary vitamin B_{12} deficiency occurs only in vegans, i.e. strict vegetarians. In our subjects, with dietary vitamin B_{12} deficiency, only 16·6% were absolute vegetarians. The others ate animal protein but only once in 1–4 weeks. Secondly, in the folic acid-deficient group all subjects ate vegetables. It is well recognized that boiling vegetables in large quantities of water and discarding the water may result in as much as 95% loss of folate. However, the Western method of cooking (boiling) vegetables is rare in Sri Lanka where vegetables are usually eaten as a 'curry'. In this preparation, the vegetables are cooked in a minimum quantity of water which is allowed to soak into the vegetables during cooking. Further, not all of these vegetables are cooked. As has been stated previously, the 'mallun' (see p. 496) is eaten almost daily and is cooked very little.

The appearance of a folate deficiency in people eating this type of diet is difficult to explain. The effect of repeated pregnancies on body folate stores undoubtedly plays a part. From the point of view of the diet, the only striking difference between that of the folic acid-deficient Ceylonese and the average Western diet is the inadequacy of animal protein, especially liver, which is hardly ever eaten by anyone in the lower-income group. Liver is one of the richest sources of folic acid and it is possible that this represents an important source of folic acid in the West. Another important possibility is a failure in the hydrolysis of the pteroylpolyglutamates in food to pteroylmonoglutamates by intestinal pteroylpolyglutamate hydrolase (conjugase). This enzyme may well be lacking in people whose intestines harbour a heavy parasitic infection or are subjected to frequent attacks of bowel infection. A related possibility is the role of conjugase inhibitors in our food. The exact mechanism of folic acid deficiency in the developing countries cannot be determined without a detailed analysis of folate contents of tropical diets and the study of intestinal conjugase and its inhibitors in food. Such studies are now in progress.

Therapeutic implications

The therapeutic implications of this study are that subjects with nutritional megaloblastic anaemia can be successfully treated with a small oral dose of vitamin B_{12} or folic acid. A single 'haematinic pill' consisting of 200 mg ferrous sulphate, 10 μg cyanocobalamin and 200 μg folic acid could be of value in circumstances in which the nutritional status cannot be improved because of poverty. The cost of such a preparation would be negligible.

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