Human Vitamin \text{B}_{12} \text{ Deficiency}

S. J. Baker

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I. Introduction

The history of the understanding of human vitamin B₁₂ deficiency begins with the demonstration by MINOT and MURPHY (1926), of the response of patients with pernicious anemia to dietary therapy. This was followed shortly afterwards by the work of CASTLE (1929), which delineated the roles of the so-called 'extrinsic' or dietary factor, and the
'intrinsic' factor which was shown to be missing in patients with pernicious anemia. The independent work of Smith (1948) and Rickes, Brink, Koniuszy, Wood and Folkers (1948), resulted in the identification and characterisation of the 'extrinsic' factor as vitamin B₁₂. During the eighteen years since the vitamin was isolated, knowledge concerning its availability, absorption and metabolic functions has greatly increased, and this in turn has led to a better understanding and characterisation of deficiency states.

This review deals specifically with human vitamin B₁₂ deficiency—its pathogenesis, its clinical and metabolic effects, and its diagnosis and treatment.

II. Daily Requirements of Vitamin B₁₂

A. Adults

The amount of vitamin B₁₂ needed daily by adults has been variously estimated to be from 10 μg (Gräsbeck, 1959) to 0.1 μg (Sullivan and Herbert, 1965). The higher values are based on the study of body vitamin B₁₂ turnover. It has been shown in turnover studies that the percentage loss per day is independent of the body stores—the less the body stores the less the total lost per day from the body, and vice versa (Bozian, Ferguson, Heyser, Meneely and Darby, 1963). The lower figure is based on the minimal amount of vitamin B₁₂ which, when given by injection, causes a suboptimal hematological response in patients with vitamin B₁₂ deficiency anemias. There is now considerable evidence that the daily injection of 1.0 μg of vitamin B₁₂ will cure vitamin B₁₂ deficiency megaloblastic anemia and cause gradual elevation of serum vitamin B₁₂ levels. It seems probable, therefore, that the daily amount of cyanocobalamin necessary to maintain reasonable body stores is in the region of 0.5 to 1.0 μg.

It is important to realise that most studies have been done under somewhat artificial circumstances using pharmacological preparations of the vitamin (cyano- or hydroxy-cobalamin), and not studying vitamin B₁₂ as eaten in the food. It may well be that the availability and retention of the vitamin from different foods varies, so that the daily minimum intake may partly depend on the type of diet. Reizenstein and Nyberg (1959) studied the absorption of labelled vitamin B₁₂ as incorporated into calf and pig liver, and concluded that this was better
absorbed than labelled cyanocobalamin. However, they used relatively large amounts of the vitamin, and SULLIVAN, HERBERT and REIZENSTEIN (1962), using physiological amounts of liverbound vitamin B₁₂ were unable to repeat this finding.

B. Children

The total daily vitamin B₁₂ requirement of infants and children is presumably less than that of adults, but no precise figures are available. However, 0.1 μg of cyanocobalamin by mouth will cure severe vitamin B₁₂ deficiency anemia in infants (JADHAV, WEBB, VAISHNAV and BAKER, 1962) and the daily requirements are presumably less than this.

The daily vitamin B₁₂ requirements of the body must normally be met either by the vitamin absorbed from food, or by calling upon body stores. When the intake and absorption of the vitamin are unable to meet the needs of the body, vitamin B₁₂ stores will be reduced and frank vitamin B₁₂ deficiency will eventually supervene. Vitamin B₁₂ deficiency may therefore arise from defective intake, defective absorption or increased demands for the vitamin, or from a combination of these mechanisms.

III. Dietary Sources and Dietary Deficiency of Vitamin B₁₂

A. Fetuses

The developing fetus in utero is entirely dependent on transplacental passage of vitamin B₁₂ from the mother. This transfer from the mother appears to occur preferentially. The serum levels in the cord blood at birth are higher than in the maternal blood (OZEDA, HELLIGER and CHOW, 1956; DUMONT, 1957; BOGER, WRIGHT and BAYNE, 1957; DIXIT, MOY, JHALA, PAREKH and RAMASARMA, 1957) and this is true even in cases where maternal serum levels are below normal (BAKER, ZIFFER, PASQUER and SOROTKA, 1958; BAKER, JACOB, RAJAN and SWAMINATHAN, 1962). The vitamin B₁₂ stores of the normal fetus at birth do not appear to have been widely studied. At term the newborn child probably has 20–30 μg of vitamin B₁₂ stored in the liver (ROSS and MOLLIN, 1957). In children born of vitamin B₁₂ deficient mothers the
liver vitamin B₁₂ may be considerably reduced and one child has been reported with megaloblastic changes in the marrow at birth (Baker, Jacob, Rajan and Swaminathan, 1962).

B. Infants

The purely breast-fed infant is dependent on its own body stores of the vitamin, together with the further supply derived from the mother’s breast milk. The level of vitamin B₁₂ in breast milk is similar to the level in the serum, therefore vitamin B₁₂ deficient nursing mothers will provide milk with a low vitamin B₁₂ content (Gerrasi and Burgio, 1962; Baker, Jacob, Rajan and Swaminathan, 1962). Children suckled by such mothers may develop a characteristic vitamin B₁₂ deficiency syndrome during the first year or two of life (Burgo, Russo and Jacono, 1956; Jadhav, Webb, Vaishnava and Baker, 1962).

C. Children and Adults

Once weaned, the individual is dependent on ingested food to supply their vitamin B₁₂ requirements. Animal products such as liver, meat, fish, egg and milk (Lichtenstein, Beloam and Murphy, 1962; Banerjee and Chatterjea, 1963) contain vitamin B₁₂ largely as the coenzyme form (Barker, Smyth, Weissbach, Toohey, Ladd and Volcantis, 1960). Although most of these are eaten in the cooked state, little work has been done on the effects of cooking (Banerjee and Chatterjea, 1963).

It has generally been held that there is no significant amount of vitamin B₁₂ in plant foods (Evans and Kilner, 1964). Some amount of vitamin B₁₂ activity can be demonstrated in root nodules of both legumes (Levin, Funk and Tiedler, 1954) and non-legumes (Kilner and Evans, 1962), but whether this represents synthesis by the plant or is the result of bacterial activity is not certain (Bond, Adams and Kennedy, 1965). However, Fries (1962) grew peas (Pisum sativum) under sterile conditions in an artificial medium and demonstrated the apparent synthesis of small amounts of vitamin B₁₂ by the plants in the absence of bacteria. The importance of these rather small amounts of vitamin B₁₂ demonstrable in plant foods, in the nutrition of vegetarians, has yet to be determined.
The bacteria in the large intestine of many animals, and of man, are able to synthesise vitamin B₁₂. Even in subjects with pernicious anemia in relapse the daily output of vitamin B₁₂ in the feces may be in the region of 5 μg (GIRDWOOD, 1950), but this material is not available to the non-coprophagic animal.

Frank dietary vitamin B₁₂ deficiency in older children and adults may be seen in people living on a poor diet (HARRISON, BOOTH and MOLLIN, 1956) and in vegetarians (PATEL, 1948; DAS GUPTA, CHATTERJEE and BASU, 1953; BADENOCH, 1954; MOLLIN and ROSS, 1954; WOKES, BADENOCH and SINCLAIR, 1955; WOKES and SMITH, 1962). Serum vitamin B₁₂ levels among vegetarians are low (WOKES, BADENOCH and SINCLAIR, 1955; MEHTA, REGI and SATOSKAR, 1964) and analysis of liver vitamin B₁₂ stores shows a marked reduction as compared with non-vegetarians. The low vitamin B₁₂ stores of many vegetarians is further confirmed by the fact that when vitamin B₁₂ absorption is interfered with—for example in tropical sprue—frank vitamin B₁₂ deficiency symptoms may develop within a few months (Baker, 1957) whereas in a person with normal body stores it may take one to five years (PITNEY and BEARD, 1955).

In spite of a very low vitamin B₁₂ intake by the largely vegetarian population in South India, pure dietary vitamin B₁₂ deficiency is an uncommon cause of megaloblastic anemia. In addition to the low dietary intake and low body stores there is usually a further precipitating factor causing interference with vitamin B₁₂ absorption (Baker, 1965).

IV. Vitamin B₁₂ Absorption — Physiology and Pathology

It is now well established that in man there are two possible mechanisms of vitamin B₁₂ absorption—one involving supraphysiological amounts, occurring in the upper part of the small intestine, and not requiring intrinsic factor (ROSS, MOLLIN, COX and UNSLEY, 1954; HEINRICH, GARBE, WHANG and WOLFGELDER, 1965) and one involving physiological amounts, which is dependent on the presence of intrinsic factor, and which occurs in the ileum (MOLLIN, BOOTH and BAKER, 1957; BOOTH and MOLLIN, 1959; OKUDA and MRRO, 1965). It is only the latter which is of importance in maintaining nutrition in normal circumstances.

Although intrinsic factor was described many years ago (Castle, 1929) there is still a great deal which is not known about it. It has even been suggested by one group of workers that intrinsic factor does not exist (Heathcote and Mooney, 1962). This claim, however, is based on inadequate evidence, and has not been confirmed by any other workers. An excellent and comprehensive review of the knowledge about intrinsic factor has been given by Glass (1963). Pure intrinsic factor has not yet been obtained, but all studies to date indicate that it is probably a mucoprotein which avidly binds vitamin B12. One preparation from human gastric juice obtained by Chosy and Schilling (1963) significantly enhanced the absorption of 1 μg of vitamin B12 in patients with pernicious anemia, at the level of 10 μg of nitrogen. In man, intrinsic factor is produced by the acid secreting parietal cells of the body of the stomach (Horademker, Arells, Wachters, Arends and Nieweg, 1964; Taylor, 1965). Secretion of intrinsic factor can be stimulated by histamine, betazole hydrochloride, methacholine, insulin and gastrin (Jefferies and Seifinger, 1965; Ardesman and Chana-ray, 1965; Irvine, 1965; Wangel and Calleender, 1965).

B. Defective Intrinsic Factor Secretion

i. Total gastrectomy

Absence of intrinsic factor secretion will result in failure of vitamin B12 absorption, and when body stores are depleted, frank vitamin B12 deficiency. Total gastrectomy removes all the intrinsic factor producing area, and if the patient survives long enough vitamin B12 deficiency supervenes (Swendseid, Halsted and Libby, 1953; Purney and Beard, 1955; Uk, 1962). Removal of all the parietal cell mass has the same effect (Wruble, Cole, Lessner, Haidr and Kals, 1964).

ii. Partial gastrectomy

Partial gastrectomy will cause reduction of intrinsic factor secretion proportional to the amount of intrinsic factor secreting area removed. The incidence of megaloblastic anemia following partial gastrectomy is probably low. MacLean (1957) found only nine cases in a review of 1550 patients, and in all of these there was gastric
atrophy in the resected part of the stomach. However, Dreller and Witts (1962), and Mollin and Hines (1964), found an incidence of vitamin B₁₂ deficiency, as measured by low serum vitamin B₁₂ levels, of 14% and 15%, respectively. Brodine, Friedman, Saenger and Will (1959) found abnormal vitamin B₁₂ absorption in four out of thirty-four patients who had had a partial gastrectomy. They suggest that defective absorption usually occurs in those patients who have had a partial gastrectomy and gastrojejunostomy performed, rather than in those that have had a Billroth I type of operation. This may be related to more extensive resection of the stomach, to the by-passing of the pylorus and the lack of stimulation of secretion from distension of the gastric antrum, to the ‘blind loop’ formed by the afferent loop, and finally to a greater likelihood of the mucosa in the gastric remnant undergoing secondary changes in the gastrectomy-gastrojejunostomy type of operation.

iii. Congenital absence of intrinsic factor

Lack of intrinsic factor secretion sometimes occurs as a rare congenital defect, with normal gastric morphology as seen by conventional microscopy, and with normal secretion of acid and pepsin (Mollin, Baker and Doniach, 1955). Unlike patients with pernicious anemia these patients have a complete absence of circulating antibodies to intrinsic factor or parietal cells (Herbert, Streiff and Sullivan, 1964; Doniach, Rott and Taylor, 1965; McIntyre, Sullivan, Jeffries and Silver, 1965). They probably have a congenital biochemical defect preventing intrinsic factor elaboration by the parietal cells. In the family reported by Waters and Murphy (1963) this was clearly an hereditary defect.

iv. Pernicious anemia

In classical pernicious anemia, as seen in adults, intrinsic factor secretion is greatly reduced or absent (Castle, 1929). This lack of intrinsic factor secretion is always associated with gastric atrophy. The literature describing the changes in the gastric mucosa has been well reviewed by Wood and Tapp (1958). Patients with pernicious anemia also have a high incidence of intrinsic factor and parietal cell antibodies (Doniach, Rott and Taylor, 1965).

It is well known that pernicious anemia tends to run in families (Wilkinson, 1949). Studies of relatives of patients with pernicious anemia show that they have an increased incidence of gastric atrophy
(CALLENDER and SPRAT, 1962) and an increased incidence of intrinsic factor and parietal cell antibodies (DONIACH, ROY and TAYLOR, 1965) as compared with the general population. This familial predisposition to pernicious anemia presumably has a genetic basis. The relatively lower incidence of the disease in the American Negro (WINTERROST, 1961) and its rarity in South India (BAKER, 1958) must presumably have a similar basis.

v. Chronic gastritis

Chronic gastritis of unknown etiology may lead to defective vitamin B₉ absorption (SHURALA and NYBERG, 1957; SHURALA, EBAMA and NYBERG, 1960; WOOD, RALSTON, NGAR and COWLING, 1964; COGHLAN, DONIACH, ROY, MOLLIN and WILLIAMS, 1965). In a given individual, the dividing line between idiopathic chronic atrophic gastritis and pernicious anemia cannot be clearly drawn with currently available techniques. Presumably they differ in the etiology of the gastric lesion.

In tropical sprue, a pernicious anemia like lesion may develop in the stomach (VAISH, SAMPATHKUMAR, JACOB and BAKER, 1965). Assay of intrinsic factor secretion in patients with sprue shows that in some cases it is markedly reduced and this may of itself lead to defective vitamin B₉ absorption quite apart from any effect of sprue on the small intestine (BAKER and RICHARDS, 1966).

Gastritis, gastric atrophy and decrease of intrinsic factor secretion may also occur in people infested with the fish tapeworm Diphyllobothrium latum (SHURALA, PALVA and NYBERG, 1964; PALVA, 1964) and in some patients with diverticulosis (MOLLIN, BOOTH and BAKER, 1957).

C. Normal Intestinal Absorption of Vitamin B₉

i. Site of absorption

The B₉-intrinsic factor complex passes down the intestine and is absorbed in the ileum. The reason why absorption occurs in the ileum rather than in the jejunum is completely unknown. The presence of specific ‘receptors’ for the vitamin B₉-intrinsic factor complex has been postulated by NIEWEG, SHEN and CASTLE (1957), HERBERT (1959c) and others, but this is only another way of saying that there is something different about the ileal as compared to the jejunal mucosal cells. The difference does not appear to be one of environment.
Removal of the jejunum in man does not lead to defective vitamin B₁₂ absorption (Booth and Mollin, 1959). In the dog, when the ileum and jejunum were transposed, normal vitamin B₁₂ absorption continued to occur in the transplanted ileum, and did not occur in the jejunum (Drapanas, Williams, McDonald, Heyden, Bow and Spencer, 1963).

ii. Role of calcium ions

In animals the adsorption of the vitamin B₁₂-intrinsic factor complex to the intestinal mucosa is ‘calcium dependent’ and is inhibited by EDTA (Herbert, 1959b). Gräbeck and Nyberg (1958) showed that vitamin B₁₂ absorption in man was decreased when the sodium salt of EDTA was given with a test dose of radioactive labelled vitamin B₁₂. Whether the removal of calcium ions by EDTA specifically affects the process of vitamin B₁₂ absorption, or whether it is a general effect on the cell, or the cell environment, is not known. Hansen (1959) showed that calcium ions are important for the maintainance of the intercellular binding substance, and possibly of the cell membrane, in the rat ileum. This suggests that the effect of deficiency of calcium ions on vitamin B₁₂ absorption may be a non-specific one.

iii. Fate of intrinsic factor

Once the vitamin B₁₂-intrinsic factor complex reaches the ileal absorptive cells the fate of intrinsic factor is unknown. Boass and Wilson (1964) in studying vitamin B₁₂ absorption in sacs of hamster ileum inferred that the vitamin B₁₂-intrinsic factor complex was itself absorbed. However, their results can equally well be explained on the basis of adsorption to the surface of the mucosal cells. Ardemann, Chanarin and Berry (1965) studied the biliary excretion of intrinsic factor and the levels of serum intrinsic factor antibodies in man, before and after the oral administration of large amounts of intrinsic factor. They could detect no evidence that absorption of intrinsic factor had occurred. This cannot be considered conclusive evidence against absorption of intrinsic factor unless it is shown that the intravenous administration of physiological amounts of intrinsic factor to such patients is followed either by detectable changes in antibody titre, or by excretion of intrinsic factor in the bile in detectable amounts—an experiment which cannot be performed until relatively pure human

¹ Wilson (Medicine 43: 669–677, 1964) subsequently suggested that the results could be explained by ‘entrainment’ of B₁₂-IF complex between the villi or microvilli.
intrinsic factor is available. It has been suggested that the finding of antibodies to intrinsic factor in patients with pernicious anemia is evidence against the absorption of intrinsic factor as 'otherwise the reticuloendothelial system would not have treated it as a foreign protein' (ArDEMAN, CHANARIN and BERRY, 1965). On the contrary the occurrence of intrinsic factor antibodies is conclusive evidence in favour of the entry of at least some molecules of intrinsic factor into the circulation (presumably by absorption) for without this there could be no antibody formation. The failure of the body to recognize the intrinsic factor antigen as 'self' (BURNET, 1959) is one which applies to all autoantibody formation, and is probably independent of the question of intrinsic factor absorption.

Whatever the fate of intrinsic factor at the cell surface, within the cell or in the body, it is a well established fact of clinical medicine that people with intrinsic factor deficiency can be kept in perfect health by the parenteral administration of vitamin B₁₂ alone. There is thus no clinical evidence to support the suggestion of an extra-intestinal role for intrinsic factor.

in. Releasing factor

A factor which releases vitamin B₁₂ from its combination with intrinsic factor has been described by several authors (UNGLEY, (1951–52); COPPER and CASTLE, 1960; HERBERT, COPPER and CASTLE, 1962; ELLENBOGEN and HIGHLEY, 1963). It has been suggested that the findings in the experiments cited, may be due to exchange of unlabelled vitamin B₁₂ in the tissue extracts with the intrinsic factor bound radiovitamin B₁₂ (DONALDSON and KATZ, 1963; HIGHLEY, STREIFF, ELLENBOGEN, HERBERT and CASTLE 1965). Studies by RAHMAN, RAOHAKRISHNAN and BAKER (1964), however, suggest that the release obtainable is greater than can be explained on the basis of unlabelled vitamin B₁₂ in the tissue extracts. The existence of otherwise a 'releasing factor' in animal intestine must still be considered as uncertain, and its presence and role, if any, in man even more uncertain.

From the time that the vitamin B₁₂-intrinsic factor complex reaches the site of absorption, till the time it appears in the blood stream there is a delay of several hours (BOOTH and MOLLIN, 1956) which presumably is occupied by the passage of the vitamin B₁₂ across the cell membrane into and through the cell, and out into the blood stream (DOSCHERHOLMEN, HAGEN and OLN, 1959).
D. Defective Intestinal Absorption of Vitamin B₁₂

Defective vitamin B₁₂ absorption in the presence of adequate dietary intake and adequate intrinsic factor production, may occur in a number of different conditions which interfere in one way or another with the process of absorption.

i. Fish tape worm infestation

In areas where the fish tape worm (*Diphyllobothrium latum*) occurs, infestation with this worm may lead to vitamin B₁₂ deficiency. This is most likely to occur when the infestation is maximal in the proximal part of the jejunum (von Bonsdorff, 1947). In addition to the effect on the stomach already referred to, the tapeworm takes up vitamin B₁₂ from the lumen of the intestine and so competes with the host for the available dietary vitamin B₁₂ (von Bonsdorff, 1957; von Bonsdorff, Nyberg and Grästorp, 1961; Nyberg, 1960).

ii. Intestinal diverticula

The syndrome of intestinal diverticula with megaloblastic anemia was apparently first recorded by Taylor (1930) and Harvey and Murphy (1933). It has subsequently been shown by a number of investigators that the anemia is due to vitamin B₁₂ deficiency secondary to defective vitamin B₁₂ absorption. This absorptive defect can be corrected by the administration of broad spectrum antibiotics (Mollin and Baker, 1955; Badenoch, Bedford and Evans, 1955; Mollin, Booth and Baker, 1957; Cooke, Cox, Fone, Meynell and Gaddie, 1963). Many case reports are of patients with multiple diverticula but the same defect can be found in patients with a single diverticulum (Goldstein, Cozzolino and Wirtz, 1963; Fulpius, Hauser and Rosner, 1965). The almost invariable response of the vitamin B₁₂ malabsorption to antibiotics suggests that it is closely related to bacterial infection, but the precise mechanisms by which the absorptive defect is produced is not clear. From animal experiments it has been suggested that the chief factor is the uptake of the vitamin B₁₂ by bacteria in the diverticulum (Donaldson, 1962). It is possible that other mechanisms may also be involved such as pH changes (Cooke, Cox, Fone, Meynell and Gaddie, 1963) or the formation of some toxic substances such as indole (Drexler, 1958), unconjugated bile salts (Dawson and Isselbacher, 1960; Donaldson, 1965) or toxins formed by *E. coli* (Booth and Heath, 1962). Paulk and
FARRAR (1964) studied a patient with extensive diverticulosis of the small intestine. They sampled the bacterial flora from the duodeno-jejunal junction, before and after tetracycline therapy. They were unable to show a quantitative difference in flora, but could show a qualitative difference in the type of E. coli recovered before and immediately after antibiotic therapy. Six months later when the absorptive defect had returned, the original type of organism was again recovered.

They showed further that there was no apparent difference in the vitamin B12 binding capacity of the organisms before and after chemotherapy, and reasonably suggest that the original strain may have produced a toxin which caused interference with absorption of vitamin B12 and other substances.

It is interesting to note that in two cases of diverticulosis described by MOLLIN, BOOTH and BAKER (1957) and in three of the cases described by COOKE, Cox, FORNIEZ, MEYNELL and GADDIE (1963) the vitamin B12 absorptive defect was improved by the administration of intrinsic factor. The obvious conclusion is that these patients were cases of pernicious anemia with coincidental diverticulosis. It is, however, also possible that the gastric lesion causing the intrinsic factor deficiency was secondary to the diverticulosis (MOLLIN, BOOTH and BAKER, 1957).

iii. Intestinal short circuits
Vitamin B12 malabsorption and vitamin B12 deficiency have been found in many subjects with short circuits of the intestinal tract in a variety of different anatomical arrangements (CAMERON, WATSON and WITTS, 1949; DODG and GRIDWOOD, 1960), gastroenterostomy (GRIDWOOD, 1956), gastro-colic fistula (FAIRLEY and KILNER, 1931; MOLLIN, BOOTH and BAKER, 1957), jejuno-jejunostomy (DONALDSON, 1965), jejuno-ileostomy (SHERMAN and MAT, 1963), ileo-ileostomy and ileo-coloanostomy (SHRALL and KAPLANEN, 1953; BUCHWALD, 1964). In some of the recorded cases the malabsorption became normal following antibiotic therapy, suggesting that the abnormality was related to an abnormal bacterial flora (HALSTED, LEWIS and GASTNER, 1956).

iv. Intestinal strictures
Strictures of the small intestine may be associated with megaloblastic anemia (BARKER and HUMMEL, 1939; CAMERON, WATSON and WITTS, 1949; THOMPSON and UNGLEY, 1955) and with defective vita-
min B12 absorption (Glass, 1956; Cooke, 1960; Booth and Mollin, 1960), and may perhaps be associated with intestinal stasis and abnormal bacterial growth such as has been shown to occur in diverticulosis.

A number of investigators have created blind intestinal colostomies in animals, particularly in rats, for the experimental study of the anemia produced by this procedure (Cameron, Watson and Witts, 1949b; Toon and Wangensteen, 1950; Donaldson, 1962). Although the surgeon can occasionally produce such a lesion in man this must be a very rare occurrence. Duodenal and jejunal diverticula are the nearest clinical approximation to these experimental states. There are a number of reports in the literature of patients with a “blind loop” syndrome and vitamin B12 deficiency, however these are almost invariably cases of diverticulosis, short circuits, or entero-anastomoses of one type or another and not true intestinal colostomies.

It is interesting to note that not all cases of diverticulosis, short circuits, small intestinal fistulae or strictures have defective vitamin B12 absorption. The reason why some have a defective absorption and others with an apparently identical anatomical lesion, do not, is unknown. It is possible that it is related to the type of bacterial flora involved, but this subject needs further detailed study.

v. Intestinal resection

Since vitamin B12 is absorbed in the ileum, resection of the ileum leads to defective absorption and the development of vitamin B12 deficiency (McIntyre, Sacks, Kreivans and Conley, 1956; Mollin, Booth and Baker, 1957; Booth and Mollin, 1959; Booth and Mollin, 1960; Dallman and Diamond, 1960; Doug and Gradwohl, 1960; Cornell, Gilde, Moody, Frey and Beal, 1961; Fonk, Cooke, Metnere and Harris, 1961; Sherman and May (1963) described a patient who had a jejuno-transverse colostomy done for weight reduction. This resulted in the exclusion of the whole of the ileum, and in vitamin B12 malabsorption. This malabsorption was subsequently partially corrected when a short length of terminal ileum was reintroduced into normal anatomical continuity.

vi. Calcium deficiency

The role, if any, of calcium deficiency in the production of clinical vitamin B12 deficiency states is not clear. Idiopathic hypoparathyroidism may be associated with defective vitamin B12 absorption which returns to normal following vitamin D therapy (Clarkson, Kowles-
sars, Hornsby and Sleisinger, 1960; Halmon, Kendall and Ogryzlo, 1962). The probable requirement for calcium ions in vitamin B₁₂ absorption suggests that the malabsorption in these cases may be related directly to the hypocalcaemia. Further studies on similar cases would be of interest.

Patients receiving sodium phytate therapy for hypercalcuria developed signs of vitamin B₁₂ deficiency, although their vitamin B₁₂ absorption tests were normal (Herbert, 1959a). The reason for the development of vitamin B₁₂ deficiency in these cases is not clear.

Gräbeck, Kantero and Siurala (1959) found that the administration of calcium to some patients with idiopathic steatorrhoea decreased the abnormality of vitamin B₁₂ absorption. Herbert (1959a) could not confirm this observation, and similarly Rosado-Rodriguez and Sheret (1961) found no improvement in the vitamin B₁₂ absorptive defect in tropical sprue on giving extra calcium.

**vii. Pancreatic disease**

Several investigators have recorded vitamin B₁₂ malabsorption in cases of pancreatic disease (McIntyre, Sacks, Kreivans and Conley, 1956; Frost, Goldwein and Kaufman, 1957; Perman, Gullberg, Reznystein, Snellman and Allen, 1960; Nieweg, Abeles, Veeger and HELLEMAN, 1962; Veeger, Abeles, Helleman and Hlweg, 1962). The vitamin B₁₂ malabsorption was not corrected by the administration of extra intrinsic factor, but in some cases it was improved by the administration of sodium bicarbonate, and/or pancreatin. It has been suggested that this is probably a pH effect (Veeger, Abeles, Helleman and Nieweg, 1962). Not all cases of pancreatic insufficiency show defective vitamin B₁₂ absorption, but it is possible that those with normal absorption may be those with less change in intestinal pH.

The precise role of pancreatic enzymes in vitamin B₁₂ absorption is not clear. In the study by Veeger, Abeles, Helleman and Nieweg (1962) the results of Schilling tests in some patients with pancreatic disease indicated better absorption when pancreatin and sodium bicarbonate were given together than when bicarbonate was given alone. It may be that pancreatic enzymes themselves in some way facilitate vitamin B₁₂ absorption. However, there are recorded cases of complete absence of pancreatic enzymes with normal vitamin B₁₂ absorption (Schwachman, Diamond, Oski and Khoaw, 1964) so the effect of pancreatin is unlikely to be a direct one.
In view of the suggestion of a pH effect it is of interest that a case of Zollinger-Ellison syndrome with greatly increased acid secretion had normal vitamin B₁₂ absorption¹. Study of other similar cases may help to throw further light on this subject.

**viii. Drugs**

Heinivaara and Palva (1964) found two cases of megaloblastic anemia in patients receiving para amino-salicylic acid (PAS) therapy for tuberculosis. These authors showed that patients receiving PAS developed a vitamin B₁₂ absorptive defect which was not corrected by addition of intrinsic factor. The defect did not appear immediately on starting PAS, but only after several weeks of therapy. A group of patients receiving PAS for a year or more showed considerable reduction in their serum vitamin B₁₂ levels as compared with a control group (Heinivaara and Palva, 1965). In view of these findings, it is strange that vitamin B₁₂ deficiency megaloblastic anemia has not been more often recorded in patients receiving PAS therapy. One reason may be the high level of body stores, but this cannot hold good for an essentially vegetarian population with initially low vitamin B₁₂ body stores such as occurs in India. This effect of PAS therapy needs further study.

**ix. Idiopathic selective vitamin B₁₂ malabsorption**

A syndrome of familial relapsing megaloblastic anemia and proteinuria has been described by a number of observers (Najman and Braun-Sil, 1952; Imerslund, 1960; Imerslund and Bjørnstad, 1963; Gräbeck, Gordin, Kantero and Kuhlback, 1960; Lamy, Besançon Loverdo and Aifit, 1961; Colle, Greenberg and Krivit, 1961; Spurling, Sacks and Ji, 1964). The vitamin B₁₂ absorptive defect in these patients is not influenced by intrinsic factor, antibiotics or prednisone. In the case reported by Colle, Greenberg and Krivit (1961) there was some evidence that absorption was improved when the vitamin was given with intestinal juice from another person, suggesting the presence of some intestinal factor(s) necessary for vitamin B₁₂ absorption. The results however were equivocal. Imerslund and Bjørnstad (1963) in 6 cases, and Spurling, Sacks and Ji (1964) in two well studied cases, could find no enhancement of absorption on administering the test dose of vitamin B₁₂ with normal intestinal juice. It therefore appears unlikely that the defect in patients with this

syndrome can be attributed to a defect of factor(s) in the succus entericus. The defect must presumably lie somewhere in the mechanism of absorption into, or transport through, the intestinal cells themselves.

Movitt, Mangum, Greer, Cohen and Porter (1963) briefly describe two patients in whose vitamin B₁₂ absorption was abnormal when tested with vitamin B₁₂ given alone or with intrinsic factor, but was corrected when given with 'succus entericus'. Resnick, Corman, London and Richter (1963) report studies in a woman who had undergone partial gastrectomy and gastroenterostomy and who showed a vitamin B₁₂ absorptive defect not corrected by intrinsic factor, gastric juice, antibiotics, calcium lactate or bicarbonate, but which was improved by administration of succus entericus. Unfortunately in these studies the authors did not try the effect of pancreatin or of the gastric juice of the donors of the succus entericus, so it is not certain that the factor which caused improved absorption originated in the small intestine. The currently available evidence suggests that the existence of some small bowel factor(s) necessary for normal vitamin B₁₂ absorption is equivocal, but the suggestion warrants further study.

**x. Non-tropical sprue or celiac disease**

Absorption of vitamin B₁₂ may be defective in patients with non-tropical sprue and celiac disease (Glass, 1953; Callender and Evans, 1955; Baker and Mollin, 1955; Mollin and Baker, 1955; Halsted, Swendseid, Lewis and Gassler, 1956; Oxenhorn, Estren, Wasserman and Adlersberg, 1958; Mollin, Booth and Chanarin, 1959; Fone, Cooke, Meynell and Harris, 1961; Cooke, Fone, Cox, Meynell and Gaddie, 1963; and Benson Kowelosar and Selsengier, 1964) and this may lead to vitamin B₁₂ deficiency anemia. The reported incidence of vitamin B₁₂ malabsorption in unselected series of cases varies from three out of seven, to six out of fifty. Studying only patients with megaloblastic anemia and idiopathic steatorrhea, Mollin, Booth and Chanarin (1959) found eight out of seventeen with a vitamin B₁₂ absorptive defect, and fifty seven out of one hundred and twelve patients with serum vitamin B₁₂ levels below 140 µg/ml. Oxenhorn, Estren, Wasserman and Adlersberg (1958) found that 20 out of 25 patients had abnormal vitamin B₁₂ absorption, but the basis on which the 25 were selected is not clear, nor is it clear whether all were cases of non-tropical sprue.

The reason for the vitamin B₁₂ absorptive defect in this disease is unknown. It is not corrected by antibiotics, but sometimes reverts to
normal on treatment with a gluten free diet. It is usually not corrected by the addition of intrinsic factor, although some subjects also have the gastric histological lesion of pernicious anemia (Mollin, Booth and Chanarin, 1959; Fone, Cooke, Metnall and Harris, 1961). The presence of a vitamin B\textsubscript{12} absorptive defect cannot be correlated with the histopathological findings on ileal biopsy (Mollin, Booth and Chanarin, 1959). The defect must presumably be related to damage to the as yet unknown mechanisms of vitamin B\textsubscript{12} absorption, caused by the toxic factor(s) in wheat gluten. This damaging effect has been shown by the direct instillation of wheat gluten into the proximal ileum of a patient with celiac disease in remission, producing a marked abnormality of vitamin B\textsubscript{12} absorption (Rubin, Brandsborg, Flick, Parmentier, Phelps and van Neff, 1960).

**xi. Tropical sprue**

Vitamin B\textsubscript{12} deficiency and defective vitamin B\textsubscript{12} absorption are frequent accompaniments of tropical sprue (Mollin, Booth and Baker, 1957; Baker, 1957 and 1958; Sherify, Perez-Santiago and Rubin, 1961; Rivera and Bernabe-Prida, 1963; Floch, Thomases, Cox and Sherify, 1963; Klieinstein, 1964). Occasionally many years after leaving the tropics, patients may present with a vitamin B\textsubscript{12} deficiency and a vitamin B\textsubscript{12} absorptive defect as the main manifestations of tropical sprue (Booth and Mollin, 1964). The incidence of vitamin B\textsubscript{12} deficiency will obviously depend on the incidence, severity and duration of the vitamin B\textsubscript{12} malabsorption and also on the vitamin B\textsubscript{12} stores of the patients prior to the onset of the disease. The reported incidence of vitamin B\textsubscript{12} malabsorption varies widely in different series from a very low percentage (Gardiner, 1958), 44\% (Baker and Rao, 1962), nearly 90\% (Baker, 1958; Sherify, Perez-Santiago and Rubin, 1963) to 100\% (O'Brien and England, 1965). This wide variation in incidence may be due to a number of factors. The criteria used in selection of cases for study is obviously important, e.g. on the basis of severe megaloblastic anemia (Baker, 1958) or on the basis of steatorrhea (Baker and Rao, 1962). The time of testing after admission to hospital is important, as in some patients the vitamin B\textsubscript{12} absorptive defect may quickly clear even without any specific therapy, while in others it may persist for a number of years (Baker and Rao, 1962). But even allowing for such differences there are variations which do not appear to be explainable on the basis of the present state of our knowledge. It is important to remember that tropical
sprue may well be a syndrome rather than a disease entity, and it is possible that differing etiological agents may be responsible for some of the observed differences.

As is the case with celiac disease, there appears to be no correlation between ileal biopsy findings and the presence or otherwise of vitamin B<sub>12</sub> malabsorption (Baker, Ignatius, Mathan, Vaish and Cheecho, 1962). The malabsorption is not usually corrected by the addition of intrinsic factor (Mollin, Booth and Baker, 1957; Baker, 1957). However, occasional cases of tropical sprue develop a gastric lesion identical with that seen in classical pernicious anemia, and if the intestinal malabsorption clears up, such cases may be practically indistinguishable from pernicious anemia (Baker and Rao, 1962; Vaish, Sampathkumar, Jacob and Baker, 1965).

In some cases of tropical sprue the administration of broad spectrum antibiotics will reverse the vitamin B<sub>12</sub> absorptive defect (Mollin, Booth and Baker, 1957; Baker, 1958; Tasker, 1961; Guerra, Wheby, Baggs and Bayless, 1964; Klipstein, 1964). Such a response is not seen in every case, and the proportion responding varies in different studies. Shehata, Perez-Santiago and Rubin (1961) did not find any improvement in six cases, whereas Guerra, Wheby, Baggs and Bayless (1964) with long term antibiotic therapy, found normalization of vitamin B<sub>12</sub> absorption in 12 out of 15 cases. Recent studies with serial Schilling tests, have shown that in some cases an observable improvement in vitamin B<sub>12</sub> absorption can be shown within 48–72 h of starting tetracycline therapy, but 5–7 days of chemotherapy may be necessary to reach the maximum effect. On cessation of chemotherapy, the vitamin B<sub>12</sub> absorptive defect may return within 1 to 6 weeks<sup>3</sup>. The improvement found shortly after starting antibiotics is presumably related in some way to the suppression of intestinal flora, and it must therefore be postulated that these same flora interfere with vitamin B<sub>12</sub> absorption. The reported normalization of vitamin B<sub>12</sub> absorption months after the start of chemotherapy is more difficult to relate to effects on intestinal bacteria, and such improvement also needs to be distinguished from that which may occur spontaneously. It may be that after removal of longstanding abnormal flora intestinal recovery is occasionally delayed. The mechanism by which the ‘abnormal flora’ interfere with vitamin B<sub>12</sub> absorption is completely unknown, although it is possible that this mechanism is the same or similar to that occurring in diverticulosis.

ROSADO-RODRÍGUEZ and SHEEHY (1961) studied the effect of additional calcium on the vitamin B₁₂ malabsorption associated with tropical sprue and could not demonstrate any improvement in absorption. Giving normal intestinal juice along with the test dose also produced no improvement in vitamin B₁₂ absorption¹.

HOGAN, SOERGEL and ARNAUD (1964) report a 'selective' vitamin B₁₂ absorption defect in a Puerto Rican, and SCHLOESSER and SCHILLING (1963) report studies on an Indian who also had malabsorption of glucose and vitamin A. Since both these patients came from areas where tropical sprue is prevalent, it seems that they should be regarded as variants of the tropical sprue syndrome. Similar cases, with vitamin B₁₂ malabsorption being the only demonstrable defect in what appears to be a variant of tropical sprue, have been seen in India².

HERR and ADAMS (1963) in South Africa studied vitamin B₁₂ absorption in 16 patients with megaloblastic anemia and found normal absorption in only 3 patients. This is of particular interest since tropical sprue has not been so far recorded in Africa. These authors suggest that the vitamin B₁₂ malabsorption may have been due to folic acid deficiency, but no other workers have confirmed this effect of folic acid deficiency and it would appear that some other explanation should be sought for the vitamin B₁₂ malabsorption in these cases.

V. Increased Demands for Vitamin B₁₂

Increased demands for vitamin B₁₂ presumably occur during periods of rapid growth, in pregnancy, and during rapid blood regeneration. In a person with normal body stores there are ample reserves of vitamin B₁₂ to meet any of these demands. When body stores are reduced the added stress may conceivably precipitate frank deficiency. The occasional case of vitamin B₁₂ deficiency anemia occurring in pregnancy (BAKER, JACOB, RAJAN and SWAMINATHAN, 1962) and in thyrotoxicosis (ZIFFER, GUTMAN, PASHER, SATYKA and BAKER, 1957) may represent examples of frank deficiency precipitated by increased demands for the vitamin. The reported 'masking' of megaloblastosis by iron deficiency (TASKER, 1959) could possibly be another example of this type of phenomenon—the administration of iron increasing the demand for other hematines.

VI. The 'Clinical' Effects of Vitamin B₃ Deficiency

The normal person who eats adequate amounts of animal protein has considerable body stores of vitamin B₃. Interference with intake or absorption of the vitamin will at first only cause a gradual reduction in body stores. Other effects will subsequently become apparent as the depletion of body stores becomes more marked. For the sake of convenience the 'clinical' and the metabolic effects of vitamin B₃ deficiency are discussed separately—although undoubtedly all the effects are bio-chemical in origin.

A. The Hemopoietic System

The most widely studied abnormality produced by vitamin B₃ deficiency is the effect on the developing red cells giving rise to the morphological change from a 'normoblastic' to a 'megaloblastic' pattern of erythropoiesis (EMRICH, 1880; REISSNER, 1958) and the subsequent production of a macrocytic anemia. Unfortunately, the diagnosis of a megaloblastic pattern of erythropoiesis is a subjective one depending on the experience and ideas of the observer. There is little problem about the recognition of the grossly abnormal cells, but there has been a lot of difference about the diagnosis, terminology and significance of minor grades of morphological change—the so-called 'intermediate megaloblasts' (FUNDENBERG and ESTREN, 1958; HERTBERT, 1959a; MOLLIN, 1960).

If serial marrows from one patient, or marrows from a number of patients are studied, it can be shown that there are all grades of morphological abnormality, from those which are only just distinguishable from normoblastic to the grossly megaloblastic. For the sake of convenience these extremes have been classified as 'grade I' and 'grade IV' megaloblastic, respectively—grades II and III being intermediate between these (Baker, 1958; KARTHEGAENI, GNANASUNDARAM and Baker, 1964). When only a single deficiency is involved the degree of megaloblastosis and the severity of the anemia tend to run parallel. When iron deficiency is also present the megaloblastic reaction may be less obvious or completely masked even in the presence of severe anemia (TASKEH, 1959a). The present understanding of the biochemical defects which may be responsible for these morphological changes in the cells is discussed later.

In addition to the abnormal cellular morphology, there is an increase in hemopoietic tissue in the marrow, a relative erythroid hyper-
plasia, and a defective maturation of red cell precursors (Asfalti, 1960). Measurements show that total erythropoietic activity is increased, but effective erythropoiesis, in terms of delivery of viable red cells into the peripheral blood is normal or reduced, indicating 'ineffective' red cell production (Finch, Coleman, Motulsky, Donohue and Rieff, 1956). It has long been recognised that patients with megaloblastic anaemia may be mildly icteric. This is the result of excessive hemolysis which has been quantitated by Ashby and radioactive chromium survival studies, plasma iron turnover, and studies of pigment metabolism (Hamilton, de Gowin, Sheehy, Janney and Ellis, 1954; Finch, Coleman, Motulsky, Donohue and Rieff, 1956; Schmid, Moeschlin and Hargi, 1964; Sheehy, 1964). Cells from a patient with vitamin B₁₂ deficiency have a shortened half life in the patients own circulation, and an even shorter half life in a normal compatible recipient, indicating the presence of an intracorporeal defect. However, there is also an extracorporeal defect as shown by the fact that normal red cells transfused to patients with vitamin B₁₂ deficiency anaemia have a shortened half life. This effect disappears after treatment with the vitamin. It has also been shown that contact of normal red cells with vitamin B₁₂ deficient plasma in vitro, shortens their half life when reintroduced to normal subjects to a degree comparable to the shortening of their survival in patients with vitamin B₁₂ deficiency (Hamilton, Sheehy and de Gowin, 1958). Whether the damage to the cells is caused directly by lack of vitamin B₁₂ in the plasma, or whether there is some other factor responsible does not seem to have been studied, although it should not be difficult to devise a suitable experiment.

In addition to the red cell abnormalities, there are also abnormalities in the polymorphonuclear white cells and in the megakaryocytes and platelets. The developing white cells in the marrow show a maturation arrest with giant forms and hypersegmentation of the polymorphs. In the peripheral blood there is often a leucopenia with hypersegmentation of the polymorphs which of itself is very suggestive of vitamin B₁₂ or folic acid deficiency. The changes in the white cells are presumably an expression of defective maturation and may have a biochemical basis similar to that responsible for the defective maturation of the red cells (Weickert, 1957). Whether there is also a shortened life span of the circulating white cells corresponding with the decreased red cell life span is not known.
Abnormal megakaryocytes may be found in the marrow (MAL-
LARME, 1948) and the circulating platelets may be reduced in number
and show morphological abnormalities. These abnormalities may be
so marked that patients with vitamin B_{12} deficiency from any cause
may present with purpura or other bleeding manifestations (PAD-
DOCK and SMITH, 1939; WENTROBE, 1961; MATHWEW, IGNATIUS,
MENAKSHAMMA and BAKER, 1964; STEFANINI and KARACA, 1966).
Platelet production and survival studies do not appear to have been
undertaken. STEFANINI and KARACA (1966) have shown abnormalities
in platelet function and chemical abnormalities as shown by depletion
of lipids, phospholipids, lecithin and phosphatidyl serine. All these
were corrected after vitamin B_{12} administration, but the mechanism
of production of these abnormalities is not known.

B. Epithelial Cells

Epithelial cells in many parts of the body may show morphological
abnormalities—particularly nuclear macrocytosis—in vitamin B_{12} defi-
ciency. Such changes have been described in cells of the buccal mucosa
(BOEN, 1957; FARRANT, 1958 and 1960), gastric and esophageal mucosa
(MASSEY and RUBIN, 1954; MASSEY and KLAYMAN, 1955; GRAHAM
and RHEAULT, 1954), nose, urinary tract and vaginal mucosa (BOODEN
T in and SPRIGGS, 1959). TEN THJEL (1963) has described megalocytic
jejunal epithelial cells in tropical sprue which became normal on treat-
ment with folic acid. SWANSON and THOMASSON (1965) have noticed
similar changes in patients with sprue, but did not correlate their find-
ings with vitamin B_{12} or folate deficiency. Similar changes in jejunal
mucosa have been noted in cases of vitamin B_{12} deficiency. These
returned to normal after treatment with physiological doses of vitamin
B_{12}. The metabolic basis for the observed changes is presumably
similar to that pertaining in the case of the developing red cells, but no
studies have been undertaken in this area.

C. Pigmentation

COOK (1944) described hyperpigmentation of the skin, tongue, and
lips in Indians in association with megaloblastic anemia, which cleared

1 CHACKO, C. J. C.; BAKER, S. J. and MATHAN, V. I., unpublished observations,
1964.
with the administration of crude liver extract. JADHAV, WENS, VAISHANAVA and BAKER (1962) described hyperpigmentation in Indian infants with a 'pure' vitamin B₁₂ deficiency. The hyperpigmentation was most marked over the extremities, particularly over the dorsum of the hands and feet, with accentuation over the terminal phalanges and interphalangeal joints. The hyperpigmentation disappeared on treatment with small oral doses of vitamin B₁₂. Similar hyperpigmentation has been described in adult Indians with vitamin B₁₂ deficiency (Baker, Ignatius, Johnson and Vaish, 1963). The metabolic disorder responsible for this hyperpigmentation is unknown. It is not related to adrenal insufficiency (Baker, Ignatius, Johnson and Vaish, 1963). Vitamin B₁₂ injected in small amounts locally has no effect—the effect of vitamin B₁₂ only being seen after oral administration or intramuscular injection. Studies of melanocyte stimulating hormone levels have not yet been undertaken. It is probable that folic acid deficiency can occasionally cause a similar type of hyperpigmentation. In one case with low normal serum vitamin B₁₂ levels the hyperpigmentation greatly decreased following physiological doses of folic acid alone. Nevertheless, this type of pigmentation appears to be much more characteristic of vitamin B₁₂ deficiency. Although a patchy pigmentation has been described in pernicious anemia (Castle and Minot, 1936; Strauss and Brokaw, 1951; Wintrobe, 1961), this particular type of hyperpigmentation has not been described in Caucasians with vitamin B₁₂ deficiency. It appears as though there is some biochemical difference between pigmented and non-pigmented skin which makes the former susceptible to vitamin B₁₂ deficiency states.

D. The Nervous System

Deficiency of vitamin B₁₂ in developing chick embryos may lead to congenital abnormalities in the central nervous system (Alexander, 1957), but there is no record of any such association in man (Baker, Jacob, Rajan and Swaminathan, 1962).

Acquired abnormalities of the central nervous system due to vitamin B₁₂ deficiency may occur at any time from the first few months of life onwards. Infants, with vitamin B₁₂ deficiency show apathy, developmental retardation or regression, pyramidal signs with spasticity and extensor plantar responses, extrapyramidal lesions with

tremors and involuntary movements and extensive cortical involvement as shown by electroencephalographic changes. All these manifestations tend to improve or are cured by vitamin B_{12} administration if given early enough (Gelin and IBorra, 1955; Burgio, Russo and Jacano, 1956; JadHAV, WebB, VarshNava and Baker, 1962). Generalised convulsions and coma may occur and may be precipitated by folic acid therapy (GerBasi, 1955; Pearson, Vinson and Smith, 1964; JadHAV and Janaki, 1965). As with adults with central nervous system lesions, if treatment with vitamin B_{12} is delayed, a stage may be reached when the lesions are irreversible, and vitamin B_{12} administration will no longer restore normal central nervous system function (Pearson, Vinson and Smith, 1964).

In adults, the earliest neurological lesion is often a peripheral neuritis manifesting as paresthesia or anesthesia of 'glove and stocking' type. Histological examination at this stage shows a marked demyelination of the peripheral nerve fibres (Greenfield, Blackwood, McMenemy, Mexton and Norman, 1959).

The most widely recognised and characteristic nervous systems lesion is the so-called 'subacute combined degeneration of the spinal cord'. HerBert (1959a) has pointed out that two of the earliest signs of cord involvement may be loss of sense of position in the index toe, and loss of vibration sense in the feet when tested with a 256 c.p.s. tuning fork. In an established case the lesions characteristically involve both posterior and lateral columns of the cord, with loss of sense of position, loss of vibration sense, ataxia and spastic paraplegia. In addition there is usually a greater or less degree of associated peripheral neuritis. An excellent and detailed description of the clinical features are given by Wilson (1955).

It is a popular misconception that subacute combined degeneration of the cord occurs only in cases of classical pernicious anemia. It is in fact a manifestation associated with severe vitamin B_{12} deficiency and may occur in any condition leading to such deficiency. Thus it has been described in patients with dietary vitamin B_{12} deficiency (Badenoch, 1954; Wells, 1958) with vitamin B_{12} deficiency following gastrectomy (Dennig, 1929; Rowlands and Simpson, 1932; Knox and Delamore, 1960; Weir and Gatrien, 1963; Zivin, 1964) with fish tape-worm infestation (WolTman and Hee, 1937), with jejunal diverticulosis (Krevans, Conley and Sacks, 1954; Badenoch, Bedford and Evans, 1955), with intestinal strictures and tuberculosis (Richmond and Davidson, 1958; Nicol, 1960), with ileocolic fistula
(Wilkinson and Leeds, 1955), with ileal resection (Best, 1959), with non-tropical sprue (Snell, 1936; Cooke, 1952; Richmond and Davidson, 1958), and with tropical sprue.1

The earliest observable lesion in the cord is a swelling of the myelin sheaths, followed by breakdown and removal of the lipid material by phagocytosis, Wallerian degeneration of the axons and finally gliosis. The lesions usually appear first and are most marked in the white matter in the mid- or lower-thoracic region of the cord. The reason for this distribution of lesions is quite unknown (Greenfield, Blackwood, McMenemy, Meyer and Norman, 1964). In adults with vitamin B12 deficiency besides the involvement of the peripheral nerves and the spinal cord, there may also be involvement of the higher centers, which may or may not be combined with peripheral nerve and cord lesions. Symptoms range from general slowing of the mental processes to marked depression, frank psychosis and finally severe dementia (Holmes, 1956; Ungo, 1957; Herbert, 1959a; Fraser, 1960; Smith, 1960). As might be expected these changes are also accompanied by electroencephalographic changes (Walton, Kiloh, Osselton and Farrall, 1954; Ungo, 1957; Smith, 1960) which at least in earlier cases are reversible following vitamin B12 therapy. In the few postmortems which have been done in such cases, small ill-defined perivascular areas of demyelination have been found in the cerebral white matter, and cellular degenerative lesions in the basal ganglia and other nuclei (Greenfield, Blackwood, McMenemy, Meyer and Norman, 1964).

The pathogenesis of the various neurological changes was long debated (Wolstein and Heck, 1937; Wilson, 1955). However, since the advent of microbiological assay techniques for measuring serum vitamin B12, it has been shown that in untreated cases the serum vitamin B12 concentration is invariably low (Mollin and Ross, 1954; Spray and Wits, 1959). Provided treatment is not too long delayed the response to vitamin B12 therapy is good (Ungo, 1957). It has been suggested that it is not so much deficiency of vitamin B12 per se, but the presence of some toxic factor acting together with the vitamin B12 deficiency which is responsible for the neurological damage. Various toxic factors have been suggested—parahydroxy-phenylpyruvic acid (Abbott and James, 1950; Sinclair, 1956), cyanide (Wokes, Badenoch and Sinclair, 1955), and methylmalonic acid (Cow and White, 1962; Edwin, Hottten, Norum, Schrumpf and

Skaug, 1965). It has long been recognised that there is no relation between the severity of the anemia and the incidence of neurological changes and this fact has been used by some as an argument in favour of the toxic factor theory (Wilson, 1955). However, this lack of correlation is probably explicable on the basis of the effects of folate acid which may prevent or cure the anemia but precipitate the cord changes (Israel and Wilkinson, 1949). Except for the special case of optic atrophy there is no evidence to support the toxic factor theory, and there seems little reason to suspect that the central nervous system changes are not just manifestations of the effect of vitamin B12 deficiency on nervous system metabolism.

Retrobulbar neuritis and optic atrophy may occasionally occur in patients with vitamin B12 deficiency (Cohen, 1936; Turner, 1940; Ungley, 1957; Hamilton, Ellis and Sheets, 1959). Unlike pernicious anemia and subacute combined degeneration of the cord, which have an equal sex incidence, the optic nerve involvement seems to be confined to males (Freeman and Heaton, 1961). This suggests some additional factor combining with the vitamin B12 deficiency to cause the optic nerve lesion. Freeman and Heaton (1961) suggest that this factor may be related to smoking. Smith (1961) further suggests that the toxic factor may be cyanide which is normally detoxified by hydroxocobalamin—a mechanism which may be defective in vitamin B12 deficiency.

The true incidence of neurological involvement in vitamin B12 deficiency is very difficult to ascertain. Published figures for the incidence in cases of pernicious anemia vary widely (Richmond and Davidson, 1958; Cox, 1962). This variation is probably due to a number of uncontrolled factors such as different populations, different observers and different criteria used in defining 'pernicious anemia' and 'neurological involvement'. Nevertheless, it appears that subacute combined degeneration of the cord is much less common in cases of vitamin B12 deficiency anemia seen in India, than it is amongst a group of pernicious anemia patients in the West. One possible explanation of this may be that vitamin B12 deficiency anemia in adults in India is usually due to tropical sprue and/or dietary deficiency, and in such cases the vitamin B12 deficiency is often not as marked as in cases of pernicious anemia, and it is very often associated with concomitant folate acid deficiency. It is well known that the administration of folate acid to a person with vitamin B12 deficiency may precipitate neurological involvement (Israel and Wilkinson, 1949; Fuld, 1950; Rich-
Mond and Davidson, 1959) and it is possible that the reverse may also be true—that folate deficiency protects the nervous system in cases of vitamin B₁₂ deficiency (Wokes and Smith, 1962).

The further elucidation of all these problems must await a fuller understanding of the role of vitamin B₁₂ in nervous system metabolism.

E. Other ‘Clinical’ Effects

Patients with vitamin B₁₂ deficiency frequently complain of glossitis (Oatway and Middleton, 1932). In one series of patients with pernicious anemia some evidence of tongue involvement was found in 70% of cases (Brown, 1949). At times glossitis may in fact be the presenting symptom occurring even before the onset of anemia or nervous system involvement (Adams, 1957). Histologically, the tongue epithelium is greatly reduced in thickness and this returns towards normal after treatment with vitamin B₁₂ (Taft, Hughes and Wood, 1958). Although the tongue changes are not specific (Kaplan, 1961) they may provide a useful clinical pointer suggesting the possibility of a vitamin B₁₂ deficiency state.

Patients with pernicious anemia may also complain of weight loss, malaise, anorexia, nausea, dyspepsia, and diarrhea or constipation (Wintroub, 1961; Cox, 1962). The exact relationship between these various symptoms and vitamin B₁₂ deficiency per se is not clear.

VII. The Metabolic Effects of Vitamin B₁₂ Deficiency

A. Folate Metabolism

The fact that the anemia of vitamin B₁₂ deficiency will respond, at least for sometime, to treatment with large doses of folic acid, suggests an interrelationship between these two substances (Vitler, Horrigan, Mueller, Jarrold, Vitler, Hawkins and Seaman, 1950). Other observations in subjects with vitamin B₁₂ deficiency have shed further light on this relationship. These observations relate to folic acid clearance studies, studies in histidine metabolism, and measurement of ‘folate’ concentrations in red cells, tissues and serum.
i. Folic acid clearance studies

Slay, Fourman and Witts (1951), and Girdwood (1953) showed that when a parenteral dose of folic acid was given to subjects with vitamin B₁₂ deficiency they excreted less *S. faecalis* active material than did normal subjects given the same dose of folic acid. This suggested tissue depletion or abnormal metabolism of folate in the vitamin B₁₂ deficient subjects. Similar conclusions were drawn from the study of plasma clearance following the parenteral administration of folic acid using the *S. faecalis* assay (Chanarin, Mollin and Anderson, 1958), or measuring the radioactivity in the plasma following a dose of tritiated folic acid (Mollin, Waters and Harris, 1962). Curiously, the plasma clearance of injected folic acid is much less rapid when measured by *L. casei* assay (Herbert and Zalusky, 1962; Mollin, Waters and Harris, 1962). It might have been thought that the persistence of *L. casei* activity, and the rapid disappearance of *S. faecalis* activity, was due to the conversion of pteroylglutamic acid into some compound not metabolically active for the latter organism but still utilisable by the former. However, the studies of Mollin, Waters and Harris (1962) with tritiated pteroylglutamic acid do not support this suggestion, as levels of tritium in the plasma disappeared much more rapidly than the *L. casei* levels, and paralleled the *S. faecalis* levels. The only way these findings can be reconciled with each other would be if the administered tritiated folic acid is rapidly taken up by the tissues, where it displaces some already existing (and therefore unlabelled), *L. casei* active, *S. faecalis* inactive, material (presumably N-5-methyl-tetrahydrofolate) into the plasma, which is then not cleared rapidly because there is already an excess of this material present in the plasma. Further work is obviously needed in this area before the results so far obtained can be adequately interpreted.

ii. Histidine metabolism

Evidence of disordered folate metabolism also comes from studies of formiminoglutamic acid (FIGLU) excretion after histidine loading—the so-called 'FIGLU test'—(Lohr, Cooperman and Teller, 1959) which show that some subjects, with what appear to be a pure vitamin B₁₂ deficiency, have an abnormal FIGLU excretion (Herbert, 1959a; Kohn, Mollin and Rosenbach, 1961; Zalusky and Herbert, 1961; Mollin, Waters and Harris, 1962; Hansen and Weinfeld, 1962; Knowles and Pranserg, 1962; Chanarin, Bennett and Berry, 1962; Villame and McCracken, 1963) and also in some cases an
increased excretion of urocanic acid (Chanarin, 1963; Davis and Kelly, 1963).

The response of the abnormal FIGLU test to therapy is of importance to an understanding of the metabolic disarrangements. When vitamin B12 deficient patients are treated with vitamin B12 the FIGLU test usually returns to normal. With small doses of vitamin B12 (1–5 μg daily) this may take several weeks, but with larger doses (1000 μg), the response is much more rapid. The response to folic acid, however, is not so clear. Knowles and Prankerd (1962) in three cases with vitamin B12 deficiency found that folic acid alone did not alter the excretion of FIGLU. It is difficult to draw conclusions from their studies as in two cases they only waited 24–48 h before giving vitamin B12. Hansen and Weinfield (1962) in two cases with high FIGLU excretion found little response to physiological doses of folate, 200–500 μg daily by injection, after 8 and 22 days, respectively. Chanarin (1963), on the other hand, treated three patients with folic acid only, and reduced their urinary excretion of histidine derivatives in the FIGLU test to normal after 3, 8 and 14 days, respectively—the last patient being treated by injection of only 20 μg of folic acid daily. Further work is obviously needed to fully delineate the response of the FIGLU test in vitamin B12 deficient patients treated with small doses of folic acid.

iii. Red cell and tissue folate concentration

Hansen and Weinfield (1962) demonstrated a fall in whole blood folate concentrations (L. casei) in patients with vitamin B12 deficiency. The concentrations rose towards more normal levels following treatment with vitamin B12, even though the patients were maintained on a low folate diet. The rise following vitamin B12 therapy was more marked if small doses of folic acid were given before starting vitamin B12 therapy. The rise in red cell folate coincided with the peak of the reticulocyte response. Herbert and Zalusky (1962) found after vitamin B12 therapy, in a patient with vitamin B12 deficiency, that the reticulocytes contained a much higher amount of folate material than the mature red cells, thus confirming the inference of Hansen and Weinfield that the increase in whole blood folate activity in such patients is due to the greatly elevated folate content of the reticulocytes. This explanation is further borne out by the finding of abnormally high red cell folate concentrations in other conditions where there are a lot of young cells circulating (Cox, Meynell, Cooke and
GADDIE, 1960). The finding of lowered red cell folate in vitamin B₁₂ deficiency and the rise with treatment with vitamin B₁₂ has subsequently been confirmed by COOPER and LOWENSTEIN (1964), MAGNUS (1965), MOLLIN and HOFFBRAND (1965) and JIEFFElR0Y, PATHARE and NORONHA (1965).

The relationship of red cell folates to plasma concentration is obscure. HERBERT and ZALUSKY (1962) found no evidence of a rise in red cell folate when folic acid was given intravenously, and suggested that mature cells were not permeable to folate. However, MAGNUS (1965) showed that subjects with vitamin B₁₂ deficiency treated with vitamin B₁₂ had an initial drop in red cell folate which reached its lowest in 24–48 hrs to be followed by a rise to normal or high concentrations. This drop was not prevented by administering folic acid with, or before, the vitamin B₁₂. It would appear that this drop in intracellular folate must either be due to egress of folate material from the cell, or to some alteration in the form of the intracellular folate making it unavailable to assay. Further study is needed in this field.

In vitamin B₁₂ deficient sheep, liver folate is reduced to very low levels (Dawbarn, Hine and Smith, 1958). GIRDWOOD (1959) has reported a similar finding in vitamin B₁₂ deficiency in man.

iv. Serum folate concentrations

A proportion of subjects with vitamin B₁₂ deficiency have been found to have high folate concentrations in the blood as measured by the serum L. casei assay (Herbert, Baker, Franke, Pasher, Sobotka and Wasserman, 1960; Herbert and Zalusky, 1962). In a detailed study of L. casei serum concentrations in subjects with vitamin B₁₂ deficiency due to pernicious anemia, WATERS and MOLLIN (1963) found little or no elevation in patients with mild vitamin B₁₂ deficiency, but in 100 patients with more severe vitamin B₁₂ deficiency (serum vitamin B₁₂ concentrations less than 80 μg/ml) the mean serum L. casei concentration was significantly elevated, the greatest elevation occurring in the least anemic group, and the most anemic group showing little or no elevation. The high concentrations of folate were reduced to normal following vitamin B₁₂ therapy. Whether the lower mean concentration in the anemic group is the result of folate deficiency producing the anemia, or whether in some way severe anemia reduces the folate concentrations, is at present a matter of conjecture. Since folic acid is cleared from the serum (as measured by S. faecalis)
more rapidly in more severely anemic cases (Chanarin, Mollin and Anderson, 1958), the severe anemia may in some way be responsible for the lower serum folate concentrations, and not vice versa.

The compound responsible for the elevated folate concentration found in L. casei assays was shown by Herbert, Larrabee and Buchanan (1962) to be similar on chromatography in four different solvent systems to N\textsubscript{5}-methyltetrahydrofolate. Jaenike, Waters and Mollin (1963) confirmed this observation. A similar compound appears to be the major form of folate in mammalian and avian liver (Donaldson and Keresztesy, 1959 and 1961; Silverman, Law and Kaufman, 1961; Noronha and Silverman, 1962). The folate material present in normal serum was also shown by Noronha and Aboobaker (1963) by fractionation on DEAE cellulose column, to be N\textsubscript{5}-methyltetrahydrofolate. Baker, Frank, Gelene and Levey (1964) studying the effects of a folic acid load on serum folates, suggest that 'principally PGA nor N\textsubscript{5}-methyl-THF, accumulates during vitamin B\textsubscript{12} deficiency'. However, reference to their results of bioautographic analysis shows that their statement refers only to material accumulating after a pteroylglutamic acid load in vitamin B\textsubscript{12} deficient subjects, and this bears no relation to the material present in the vitamin B\textsubscript{12} deficient subjects in the natural or unloaded state, which has a different Rf value. On the evidence available it would appear that both the folate material present in normal serum, and that which causes high L. casei values in vitamin B\textsubscript{12} deficiency probably is N\textsubscript{5}-methyltetrahydrofolate or a closely related compound.

In the conversion of homocysteine to methionine by a vitamin B\textsubscript{12} requiring E. coli mutant it has been shown that the reaction

\[
\text{homocysteine} + \text{N}\textsubscript{5}-\text{methyltetrahydrofolate} \rightarrow \text{methionine} + \text{tetrahydrofolate}
\]  

(1)

requires a vitamin B\textsubscript{12} containing enzyme (Hatch, Larrabee, Cathou and Buchanan, 1961; Larrabee, Rosenthal, Cathou and Buchanan, 1961 and 1963; Foster, Jones and Woods, 1961; Kisliuk, 1961; Takeyama, Hatch and Buchanan, 1961). A similar system has been demonstrated in mammalian liver (Sakami and Ukstins, 1961; Loughlin, Elford and Buchanan, 1964; Kerwar, Mangum, Scrimgeour and Huennekens, 1964) but not, so far, in man.

In an attempt to explain the accumulation of N\textsubscript{5}-methyltetrahydrofolate in the plasma of vitamin B\textsubscript{12} deficient patients, Herbert and Zalusky (1962) suggested that the vitamin B\textsubscript{12} deficiency led to a
deficiency of the vitamin B₁₂ containing enzyme necessary for reaction (1) with subsequent 'pile-up' of N⁵-methyltetrahydrofolate. This seemed a reasonable explanation provided, as Johns and Bartino (1965) point out, it is also assumed that this is the major pathway of N⁵-methyltetrahydrofolate metabolism. However this hypothesis does not adequately explain the finding of a reduced concentration of N⁵-methyltetrahydrofolate in the red cells and liver in vitamin B₁₂ deficiency states referred to above. Since the total amount of N⁵-methyltetrahydrofolate in the tissues is very much greater than in the plasma, it in fact appears as though the total amount of N⁵-methyltetrahydrofolate in the body is reduced in vitamin B₁₂ deficiency, rather than there being any 'pile-up' of this material. The reason for this body depletion is not clear. The depletion of body folate which occurs in vitamin B₁₂ deficient sheep is not due to reduced folate intake (Dawbarn, Hins and Smith, 1958) and many patients with vitamin B₁₂ deficiency also appear to have an adequate folate intake. There is no evidence that folate absorption is interfered with in vitamin B₁₂ deficiency, nor that there is an increased urinary excretion of folate (Anderson, Belcher, Chanarin and Mollin, 1960). It would appear that the body depletion must either be due to excessive loss, for example in the bile (Baker, Kumar and Swaminathan, 1965) or, as seems more probable, due to increased metabolic utilisation in some, presumably non-vitamin B₁₂ dependant pathway.

Tetrahydrofolate is necessary for the metabolism of formimino-glutamic acid to glutamic acid (Bakker, Silverman and Daft, 1951; Miller and Warlsch, 1956 and 1957; Tabor and Rabinowitz, 1956). If it is assumed that the major route for formation of tetrahydrofolate is via N⁵-methyltetrahydrofolate, then Herbert's hypothesis might explain the finding of a raised FIGLU excretion in many cases of vitamin B₁₂ deficiency, in that the block in conversion of N⁵-methyltetrahydrofolate to tetrahydrofolate, might result in a relative deficiency of the latter. However, since the metabolism of histidine takes place largely in the liver, the accumulation of the products of histidine metabolism in vitamin B₁₂ deficiency may merely represent one aspect of the demonstrated liver folate deficiency, rather than a specific block in N⁵-methyltetrahydrofolate metabolism.

Knowles and Pranker (1962) have suggested that the abnormalities in the FIGLU test may be due to impaired activity of formiminotransferase. There is, however, no evidence to support this hypothesis and Ohara, Chiba, Iyo and Takahashi (1964) found only
a very slight decrease in formiminotransferase activity in the liver of vitamin B₁₂ deficient rats. Work in rats is not necessarily applicable to man, but an effect of vitamin B₁₂ deficiency on formiminotransferase in humans must be considered as unlikely unless further evidence is forthcoming.

Currently available knowledge regarding the metabolic interrelationships of vitamin B₁₂ and folic acid is obviously inadequate to explain all the accumulated observations in this area.

B. Aminimidazolecarboxamide Excretion

LUNBY and COOPERMAN (1962) showed that the excretion of aminimidazolecarboxamide (AIC) in the urine was raised in patients with vitamin B₁₂ deficiency but not in patients with folic acid deficiency. In the vitamin B₁₂ deficient group excretion returned to normal after vitamin B₁₂ therapy. They therefore suggested that the reaction

\[
\text{AIC ribonucleotide} \rightarrow \text{N⁵⁺-formyltetrahydrofolate} \rightarrow \text{inosinic acid} \rightarrow \text{tetrahydrofolate}
\]  

is vitamin B₁₂ dependent.

HERBERT, STREIFF, SULLIVAN and McGEER (1964a and b) and MIDDLETON, COWARD and SMITH (1964) confirmed the finding of increased AIC in the urine of patients with vitamin B₁₂ deficiency, but also found it in patients with folate deficiency. On the basis of the folate cycle this increased excretion of AIC in vitamin B₁₂ deficiency could be explained on a lowering of tetrahydrofolate concentration leading to a deficiency of N⁵⁺-formyltetrahydrofolate, thus leading to decreased formation of inosinic acid and the relative accumulation of AIC ribonucleotide (reaction 2) and hence of AIC (LOUGHELD, ELFORD and BuchanAn, 1964). That this may not be the correct explanation is suggested by the work of HERBERT, STREIFF, SULLIVAN and McGEER (1964a) who loaded vitamin B₁₂ deficient subjects with AIC and found a smaller than normal recovery of AIC in the urine. They point out that this does not confirm the idea of a metabolic block in purine metabolism in vitamin B₁₂ deficiency, but rather suggests a more rapid purine turnover, possibly related to the hyperactivity of the bone marrow. This suggestion is further strengthened by their finding of a similar situation in relation to AIC in a case of haemolytic anaemia with normal serum vitamin B₁₂ and folate concentrations.
The morphologic abnormalities which constitute megaloblastosis of the developing red cells, and the changes noted in body epithelial cells, are presumably connected with abnormalities of ribonucleic acid (RNA) and/or deoxyribonucleic acid (DNA) synthesis.

The ratio of RNA to DNA in the developing red cells is increased in both vitamin B<sub>12</sub> and folate deficiency. This ratio tends to return towards normal with treatment (White, Leslie and Davidson, 1953; Glaser, Muller, Jerrold, Sakurai, Will and Vittler, 1954). Studies on the uptake of labelled precursors by human marrow cultures from patients with vitamin B<sub>12</sub> deficiency indicate that the defect responsible is mainly one of DNA rather than of RNA synthesis (Thomas and Lochte, 1958; Williams, Choisy and Schilling, 1963; Schmid, Moesslin and Hargi, 1964; Beck, 1964). The defect in uptake of labelled precursors can be corrected by the addition of hydroxocobalamin in vitro. In L. leichmannii it has been shown that vitamin B<sub>12</sub> in the form of 5,6-dimethylbenzimidazolylcobamide co-enzyme is necessary for the conversion of ribosyl nucleotides to deoxyribosyl nucleotides (Blakely and Barber, 1964; Abrams and Duraiswamy, 1965; Fukui, Tamao, Kato and Shimizu, 1965; Beck and Hardy, 1965). It may well be that this vitamin B<sub>12</sub> co-enzyme will prove to be necessary in man for normal DNA production, and deficiency of vitamin B<sub>12</sub> lead to deficiency of the enzyme and hence to defective DNA synthesis.

Several investigators have studied the clinical response of patients with vitamin B<sub>12</sub> deficiency megaloblastic anemia to therapy with varying results (Killman, 1964a). Killman in extending this work, gave larger doses of thymidine as a continuous intravenous infusion and obtained partial hematological responses in five patients with pernicious anemia. Killman (1964b) also studied the uptake of H<sup>3</sup>-thymidine by bone marrow cultures, and the effect on this of deoxyuridine. In normoblasts the deoxyuridine inhibited the H<sup>3</sup>-thymidine uptake, but this effect was not seen in megaloblasts from vitamin B<sub>12</sub> deficient patients. These studies definitely suggest an impairment of the conversion of deoxyuridilate to thymidilate in vitamin B<sub>12</sub> deficiency, but do not define whether this is due to a direct or an indirect effect of vitamin B<sub>12</sub> deficiency. However, Wabra and Friedkin (1962) have conclusively shown that in E. coli the conversion of deoxyuridilate to thymidilate can be represented as follows:
deoxyuridilate $\rightarrow$ $^{N_9, N^{10}}$-methylene tetrahydrofolate $\xrightarrow{Mg^{++}}$ thymidilate $\rightarrow$ dihydrofolate

There was no evidence to suggest the involvement of any vitamin B$_{12}$ compound or of $^{N_9}$-methylene tetrahydrofolate in this reaction. Although results from bacterial studies are not necessarily applicable to man, the balance of evidence at present available would suggest that any interference with thymidilate synthesis in vitamin B$_{12}$ deficiency is a secondary effect, due to decrease in the amounts of $^{N_9, N^{10}}$-methylene tetrahydrofolate available for reaction (3).

**D. Lipid Metabolism**

White (1962) found a ten to eighteen fold increase in the excretion of methylmalonic acid in the urine of vitamin B$_{12}$ deficient patients as compared with normal subjects. Cox and White (1962) confirmed and extended these observations and demonstrated that the high methylmalonic acid excretion was not affected by the administration of folie acid, but returned to normal limits following the administration of 1000 $\mu$g of hydroxycobalamin. In another case 25 $\mu$g of cyanocobalamin was given with a good hematological response, but with the persistence of a high methylmalonic acid excretion which was subsequently brought to normal only by the administration of 1000 $\mu$g of hydroxycobalamin. The finding of increased excretion of methylmalonic acid in vitamin B$_{12}$ deficiency in man has subsequently been confirmed by other workers (Barnes, Young, Millman, Kahn and Williams, 1963; Kahn, Williams, Barnes, Young, Shafer, Vivacqua and Braupke, 1965; Gioglio and Plaut, 1965). Barnes, Young, Millman, Kahn and Williams (1963) obtained a hematological response with small doses of vitamin B$_{12}$, but noted no reduction in methylmalonic acid excretion until large intramuscular doses of the vitamin were given. This necessity for large amounts of vitamin B$_{12}$, as compared with the amount needed for a hematological response, suggests that in vitamin B$_{12}$ deficiency states the requirements of vitamin B$_{12}$ for red cell production are met before those needed for this reaction. The changes in methylmalonic acid excretion in vitamin B$_{12}$ deficiency in man are presumably related to the observed requirement for coenzyme B$_{12}$ in the conversion of methylmalonyl CoA to
succinyl CoA in Propionibacterium shermanii (Stadman, Overath, Eggerer and Lynen, 1960) in Ochromonas malhamensis (Arnein and White, 1962), in rat liver (Gurnani, Mistry and Johnson, 1960; Stern and Friedman, 1960), and sheep liver (Marston Allen and Smith, 1961). It has subsequently been shown in humans with vitamin B₁₂ deficiency that there is also an increased excretion of propionic acid. This does not return to normal with folic acid therapy, but does so with vitamin B₁₂ therapy (White, 1965).

It has been suggested that the high levels of methylmalonic acid in vitamin B₁₂ deficiency may have some relation to neurological damage (White and Cox, 1964), but there is no evidence to support this suggestion.

In addition to the disorders of propionic and methylmalonic acid metabolism, there is other evidence of disturbed lipid metabolism as shown by the demyelination in subjects with neurological manifestations, and the changes in the lipid content of the platelets (Stefanini and Maracca, 1966). The metabolic interrelationships, and the precise role of vitamin B₁₂ deficiency in the production of these various disorders have yet to be explored.

E. Other Metabolic Effects

A number of investigators have studied various red cell enzymes in vitamin B₁₂ deficiency. These observations have been reviewed and extended by Vuorio (1963) and the reader is referred to this work for further details.

There are several reports on the levels of erythrocyte reduced glutathione in vitamin B₁₂ deficiency (Lawrence, 1965). The concentration of reduced glutathione was raised in more severely anemic cases and fell with treatment. On the other hand, Swarup, Ghosh, Bannerjee and Chatterjera (1965) found low levels that rose with treatment. Changes have also been found in the mineral content of the red cells in vitamin B₁₂ deficiency particularly sodium, potassium, magnesium and zinc (Valborg, Holt and Brown, 1965).

Serum alkaline phosphatase concentrations are lowered in vitamin B₁₂ deficiency anemia, but not in other anemias, and return to normal with treatment (Dommelen and Klaassen, 1964). Serum lactic acid dehydrogenase has been reported as being increased up to eighty-fold in vitamin B₁₂ deficiency anemia (Goldfarb and Papp, 1963).
The significance of these and various other biochemical abnormalities which have been postulated from time to time, is difficult to assess in the present state of our knowledge of the metabolic functions of vitamin B₁₂, and an understanding of them must await further developments.

VIII. The Diagnosis of Vitamin B₁₂ Deficiency

A. Clinical History

A careful enquiry into dietary habits may suggest that an individual could be suffering from vitamin B₁₂ deficiency due to an inadequate intake in the diet—the breast fed child, the strict vegetarian, the food fadist, etc. A history of chronic diarrhea, or of previous gastrointestinal surgery, may suggest the presence of a malabsorption syndrome producing a defect in vitamin B₁₂ absorption. A history of a similar disorder in other members of the family may be present in cases of pernicious anemia.

Patients with a mild vitamin B₁₂ deficiency may have no symptoms, or may only complain of lethargy or malaise which can easily be dismissed by the physician as neurasthenic in origin. As the effects of vitamin B₁₂ deficiency become more marked, other non-specific symptoms such as glossitis, anorexia and other gastro-intestinal symptoms, exertional dyspnea and other symptoms referable to anemia, may develop. Involvement of the nervous system—peripheral nerves, spinal cord or higher centers—especially when combined with other symptoms, may produce a clinical history very suggestive of vitamin B₁₂ deficiency.

B. Physical Examination

In many patients there are no distinctive physical signs of vitamin B₁₂ deficiency. The presence of anemia, with glossitis and a mild degree of icterus, suggest a possible diagnosis of megaloblastic anemia.

The finding of characteristic neurological involvement is strong presumptive evidence of a vitamin B₁₂ deficiency state, although similar signs may be seen in ‘nutritional myelopathies’ and other conditions (Wilson, 1965).
In Indian subjects the presence of recently acquired hyperpigmentation, especially when present on the dorsum of the terminal phalanges and over the interphalangeal joints of the fingers, is very suggestive of vitamin B_{12} deficiency.

C. Blood and Bone Marrow Morphology

Examination of the peripheral blood and bone marrow enables a diagnosis of megaloblastic anemia to be made, but does not permit of differentiation between vitamin B_{12} and folate deficiency. In the peripheral blood the characteristic changes are macrocytosis, anisocytosis and poikilocytosis of the red cells, neutropenia, with hypersegmentation of the polymorphs, and thrombocytopenia. The earliest detectable change in the peripheral blood may be the hypersegmentation of the polymorphs. The diagnostic importance of looking for polymorphonuclear hypersegmentation has been re-emphasised by Herbert (1959a). The presence of megaloblasts in the bone marrow is practically diagnostic of vitamin B_{12} or folate deficiency—a notable exception being the megaloblastosis associated with Di Guigliaim’s disease (Baldini, Fudenberg, Fukutake and Dameshek, 1959). However, the absence of megaloblasts from the bone marrow does not exclude the presence of vitamin B_{12} deficiency. The megaloblastosis may be masked by the presence of severe iron deficiency (Tasler, 1959), disorders of haemoglobin synthesis (Chanarin, Dacie and Mollen, 1959) and by therapy with folic acid. Moreover considerable tissue depletion of vitamin B_{12}, as represented by liver stores, is possible without any observable effect in the bone marrow.

The morphological changes occurring in other body cells are seldom employed in clinical diagnosis, although to the alert observer they may give a lead to the diagnosis in a previously unsuspected case of vitamin B_{12} deficiency.

D. Assay of Vitamin B_{12} Concentration

1. Serum concentration

Measurement of the concentration of vitamin B_{12} in the serum can be carried out by microbiological assay using a vitamin B_{12} dependent organism. The most commonly employed are Euglena gracilis var. bacillaris (Ross, 1952), Euglena gracilis Z strain (Hutner, Bach and
ROSE, 1956), Lactobacillus leichmannii (ROSENTHAL and SABRETT, 1952), E. coli (GROSSWICZ, AKOVITCH and RACHMILEWITZ, 1954), and Ochromonas malhamensis (FORD, 1953). Ochromonas is the most specific organism in its growth response. Euglena is a little less specific but more sensitive than Ochromonas, but has the disadvantage of requiring a five day growth period. L. leichmannii and E. coli require shorter growth periods but are less specific. ANDERSON (1964) has studied the Euglena assay extensively, and by minor modifications has greatly improved the recovery, and the reproducibility of the results.

Measurement of serum levels may also be carried out with radioactive vitamin B12 using the principle of saturation analysis (BARAKAT and EKINS, 1961; ROTHENBERG, 1961; GROSSWICZ, SOITZENI and MERZBACH, 1962). Although radioisotopic methods have not yet gained wide acceptance, the use of protein coated charcoal to separate free and bound vitamin B12 (LAI, GOTTLEIB, WASSERMAN and HERBERT, 1965) has greatly simplified the procedure and made it possible to use it for routine analysis. The normal range of serum levels varies slightly from laboratory (MOLLIN and ROSE, 1957; ANDERSON, 1964) depending on a number of variables including the method of assay and the type of population studied. In uncomplicated vitamin B12 deficiency, levels below 100 μg/ml are definitely abnormal and levels between 100 and 140 μg/ml are probably abnormal (MOLLIN, 1960). In the presence of folate deficiency however serum vitamin B12 levels may be depressed without necessarily denoting the presence of frank vitamin B12 deficiency. When such cases are treated with folic acid the serum vitamin B12 levels may rise to normal limits (NARAYANAN, SHENOY and RAMASARMA, 1957; MOLLIN and ROSE, 1957; HIFT, 1963). However, if folic acid is continued the vitamin B12 levels may fall again to lower levels. This elevation of serum levels does not follow any demonstrable increase in absorption, and it is probably related to increased mobilisation from the tissues (JOHNSON, SWAMNATHAN and BAKER, 1962). These findings make serum vitamin B12 levels in the presence of folate deficiency difficult to interpret. This is especially so for vitamin B12 levels in the range 80–140 μg/ml.

ii. Liver concentrations

Since the liver is the main storage organ of vitamin B12, estimation of the vitamin B12 concentration of liver tissue is a valuable research procedure and an excellent way of determining an individual's vitamin
Baker Human Vitamin $B_{12}$ Deficiency

$B_{12}$ stores (DROUT, WOLFF, KARLIN-WEISSMAN and RAUER, 1951; GIRDWOOD, 1952; MOLLIN and ROSS, 1957; SWENDEK, HVLBOHL, SCHICK and HALSTED, 1957; NELSON and DOCTOR, 1958; JHALA and GADGET, 1960; PENNEY and ONISTI, 1961; JOSKE, 1963; KELLY and DAVIES, 1965). However, because of the inherent risks of liver biopsy, it is never likely to become a routine diagnostic procedure.

E. Tests for Metabolic Breakdown Products

The increased excretion of methylmalonic acid found in many cases of vitamin $B_{12}$ deficiency appears to be specific for this deficiency, and does not occur in folic acid deficiency (WHITE, 1965). The original method for methylmalonic acid estimation was too cumbersome for routine use (WHITE, 1962), however, the colorimetric method described by GIORGIO and PLAUIT (1965) is simpler and may make the test more widely available.

Most workers have found that the FIGLU test cannot distinguish between vitamin $B_{12}$ and folate deficiency. The test has recently been modified by LOFTY and COOPERMAN (1965) who claim that by administering the histidine in three doses of five grams each and measuring the urinary excretion of FIGLU over a 24 hour period, subjects with vitamin $B_{12}$ deficiency will give a negative result (less than 35 mcg/m/24 hrs) and subjects with folate deficiency a positive result. Further experience with this modified test is needed before its reliability and usefulness can be assessed.

FISH, POLLYCOVE and FRIESCHMEN (1963) gave $^{14}$C labelled histidine to patients with megaloblastic anaemia and studied the subsequent urinary and pulmonary excretion of the labelled carbon. They found a normal pattern in vitamin $B_{12}$ deficiency, and a marked decrease in excretion in folate deficiency. They suggest that this test may be superior to the FIGLU test as it does not involve an unphysiological histidine load. This is undoubtedly an interesting research tool, but it is unlikely to become widely adopted as a routine test.

F. Tests of Vitamin $B_{12}$ Absorption

Tests of absorption using radioactive labelled vitamin $B_{12}$ have become an indispensable tool in the diagnosis of vitamin $B_{12}$ malabsorption. In one form or another these tests are widely used in routine
clinical diagnosis. Absorption of an orally administered dose of vitamin B₁₂ may be estimated by measuring the total fecal excretion over a period of five or more days (HINLE, WELCH, SCHARF, MEACHAM and PRUSOFF, 1952); the fecal excretion in a single fecal sample using a double isotope technique with a non-absorbable marker (GANATRA, SUNDARAM, DESAI and GAITONDE, 1963); the hepatic uptake of radioactivity (GLASS, 1954), the hepatic uptake using a double isotope technique (WEISBERG and GLASS, 1965), the urinary excretion after a flushing dose of unlabelled vitamin B₁₂ (SCHELLEN, 1953), the rise in serum radioactivity (BOOTH and MOLLIN, 1956), or the whole body retention of radioactivity (HEINRICH, GABBE, WIAHANG and WOLFSTELLER, 1965).

When malabsorption is demonstrated the test can be repeated with added intrinsic factor to see if this improves absorption. Alternatively intrinsic factor-bound vitamin B₁₂ and free vitamin B₁₂ can be differentially labelled and the absorption of the two forms studied in one test (KATZ, DIMASE and DONALDSON, 1963). For a correct interpretation of absorption results the dose employed and the mode of expression of the result are important (BAKER and MOLLIN, 1955). It is also important to realise that the demonstration of a vitamin B₁₂ absorptive defect does not necessarily imply vitamin B₁₂ deficiency and vice versa.

The literature on vitamin B₁₂ absorption tests has been extensively reviewed by DOSCHERHOLMEN (1965).

It should be remembered that most tests of vitamin B₁₂ absorption have been carried out with radioactive cyanocobalamin, whereas most naturally occurring vitamin B₁₂ is probably in the form of the vitamin B₁₂ coenzyme (BARKER, SMITH, WEISSBACH, TOOHY, LADD and VOLCANI, 1960). HEINRICH and GABBE (1964) have shown that while cyanocobalamin and vitamin B₁₂ coenzyme are equally well absorbed in the rat, in man the coenzyme form is considerably less readily absorbed. In view of this finding it needs to be shown whether or not the absorption of the two compounds run parallel. At the present time cyanocobalamin because of its greater stability and ready availability is the compound used almost exclusively for absorption studies.

G. Tests of Gastric Function

i. Acid secretion

The augmented histamine test (KAT, 1963) provides useful evidence of the functional state of the gastric mucosa. While congenital
absence of intrinsic factor secretion can occur in young people in the presence of free acid (MOLLIN, BAKER and DONIACH, 1955), in an adult the diagnosis of defective intrinsic factor secretion should not usually be made unless histamine-fast achlorhydria is present. Conversely achlorhydria can be found in chronic gastritis even where there is adequate intrinsic factor secretion. The test, therefore, is only of limited value in the diagnosis of possible intrinsic factor deficiency.

ii. Gastric biopsy

Gastric biopsy (WOOD and TAFT, 1958) enables the histological state of the gastric mucosa to be studied. The presence of a histologically normal gastric mucosa, makes the presence of intrinsic factor deficiency most unlikely, except in the rare cases of congenital absence of intrinsic factor secretion. Adult pernicious anemia is always associated with atrophic gastritis, but atrophic gastritis can also be found in patients with adequate, though possibly reduced, intrinsic factor secretion (WOOD, RALSTON, UNGAR and COWLING, 1964). This examination therefore has the same limitations of interpretation as the augmented histamine test meal.

iii. Intrinsic factor secretion

Gastric secretion of intrinsic factor can be assumed to be normal when an orally administered test dose of labelled vitamin B₁₂ is normally absorbed. Conversely when there is malabsorption of vitamin B₁₂ corrected by intrinsic factor it can be assumed that there is defective intrinsic factor secretion. However, when vitamin B₁₂ malabsorption is not corrected by added intrinsic factor, the normality or otherwise of intrinsic factor secretion cannot be inferred. Before the advent of radioactive vitamin B₁₂, it was necessary to test for intrinsic factor activity by feeding the gastric juice under study, together with extrinsic factor, to a person with pernicious anemia in relapse and observe the hematological response (CASTLE, 1929). With the advent of radioactive vitamin B₁₂ and an understanding of the stoichiometric relationship between vitamin B₁₂ and intrinsic factor (BAKER and MOLLIN, 1955), intrinsic factor assay in vitro became easier. The finding of apparently specific intrinsic factor antibodies in the sera of some pernicious anemia patients has made possible the development of a relatively simple in vitro assay for intrinsic factor (ABELS, BOUMA and NURWEG, 1963; ARDEMAN and CHANARIN, 1963; GOTTLOB, LAM, WASSERMAN and HERBERT, 1965). The specificity of these assays depends on the
specificity of the particular antibody employed. In all studies up to the present this has been assumed rather than proved. Nevertheless this test is capable of giving useful information regarding the intrinsic factor secretory status of an individual, and indirectly, of the capacity of the subject to absorb vitamin B₁₂.

H. Other Tests

Barium meal examination, with particular reference to the small intestine, is an important part of the investigation of any person suffering from vitamin B₁₂ deficiency associated with gastro-intestinal disease. Gastric atrophy (Laws and Pyman, 1960a), diverticulosis, short circuits, strictures, signs of primary malabsorption or of other disease of the intestine may be found (Anderson, Astley, French and Gerrard, 1952; Marshall and Elia soph, 1957; Paterson and Baker, 1958; Laws and Pyman, 1960b).

Other tests such as jejunal biopsy and tests of intestinal function such as fat, xylose and vitamin A absorption are important in the study of patients with vitamin B₁₂ malabsorption in order to elucidate the precise nature of the intestinal defect responsible for the malabsorption, but they do not help in the diagnosis of vitamin B₁₂ deficiency per se.

I. Therapeutic Trials

The final proof of the presence of vitamin B₁₂ deficiency anemia rests on the demonstration of an adequate response to physiological doses of vitamin B₁₂. It is important to use physiological doses, as larger doses of vitamin B₁₂ may bring about a hematological response in cases of folic acid deficiency (Zaliszyk, Herbert and Castle, 1962). If 1 μg of vitamin B₁₂ is given daily, by injection, patients with vitamin B₁₂ deficiency anemia will show a good hematological response, and patients with folate deficiency megaloblastic anemia will show no response. Conversely patients with folate deficiency megaloblastic anemia will respond to 100–200 μg of folic acid, whereas this dose will not produce any response in pure vitamin B₁₂ deficiency (Hansen and Weinfield, 1962; Herbert, 1963). When there is a combined vitamin B₁₂ and folate deficiency, treatment with small doses of vitamin B₁₂ may produce no response, or only a partial response, until folate supplementation is also given. In such cases the response to a thera-
IX. The Treatment of Vitamin B₁₂ Deficiency

A. Initial Therapy

In patients with established vitamin B₁₂ deficiency, initial therapy should aim at overcoming the deficiency and building up body stores. Whatever the cause of the deficiency, parenteral therapy is always preferable at the beginning of treatment. The initial therapy schedule for adults recommended by different workers varies widely from 100 to 1000 µg daily, every second or third day or even weekly. Although, theoretically, the larger and more frequently administered doses may build up body stores more quickly, and to a higher level, there is little clinical indication that they are superior to the smaller and less frequently administered doses.

When neurological involvement is present, larger and more frequent doses are probably advisable, although there is little evidence that such therapy exerts a more beneficial effect on the outcome.

B. Maintenance Therapy

Patients suffering from dietary deficiency of vitamin B₁₂ may be maintained in normal health, either by changing their diet to include more foods containing natural supplies of the vitamin, or by giving oral vitamin B₁₂ supplements.

In patients with permanent deficiency of intrinsic factor secretion, maintenance therapy can theoretically be given orally, either as physiological amounts of vitamin B₁₂ combined with an intrinsic factor preparation, or in supraphysiological amounts which can be absorbed without the addition of intrinsic factor (Ross, Moeller, Cox and Ungley, 1954; Chalmers and Shinton, 1958; Brody, Estren and Wasserman, 1959). However, treatment with heterologous intrinsic factor may not be successful for long term therapy owing to the development of antibodies to the intrinsic factor (Schwartz, Lous and Meulengracht, 1957; Killander, 1958).

In patients with intestinal malabsorption of vitamin B\textsubscript{12}, maintenance therapy will need to be continued as long as the absorptive defect persists. In some, the defect may be temporary as in patients with tropical sprue (Baker, 1958) or with surgically correctable intestinal lesions. In others the defect is permanent and therapy must therefore be life long. In these patients oral treatment with physiological amounts of the vitamin, with or without intrinsic factor, will not be effective. It is possible, that, as in patients with pernicious anemia, supraphysiological amounts of vitamin B\textsubscript{12} may be absorbed by diffusion.

In all patients needing prolonged treatment, apart from those with dietary deficiency, parenteral maintenance therapy is preferable. Injections of 200 to 1000 \textmu g of vitamin B\textsubscript{12} may be given once a month, or with the larger doses, once in two months. Therapy should be controlled by ensuring normal hematological values and morphology. Where possible serum vitamin B\textsubscript{12} concentrations should be checked from time to time and the concentration in the patient's serum kept within the normal range.

Hydroxycobalamin is better retained in the body than cyanocobalamin (Killander and Schilling, 1961; Glass, Skog, Lee, Jones and Hardy, 1962; Herbert, Zalusky and Skoggs, 1963; Withney and Kilpatrick, 1964; Bourne, Bottomley and Israels, 1964; Chalmers and Shinton, 1965) but its superiority in therapy is only marginal (Heinrich, Garbe, Wolfsteller and Lindor, 1965; Adams and Kennedy, 1965) so that parenteral therapy may be given either as cyanocobalamin or hydroxycobalamin. Preparations of vitamin B\textsubscript{12} with retarded absorption have been developed in an endeavour to decrease the frequency at which injections for maintenance therapy have to be given (Schwartz, Bastrup-Madsen, Nørregaard and Kristensen, 1962; Meuliengracht, 1963; Gough, Israels and Bottomley, 1964) but so far these have not achieved any degree of popularity.

X. Conclusion

In the decades that have elapsed since the original work of Minor and Murphy and Castle, great strides have been made in our knowledge of vitamin B\textsubscript{12}, and of the etiology and the effects of vitamin B\textsubscript{12} deficiency in man. Investigators from many different disciplines—nutritionists, hematologists, gastroenterologists, neurologists, patho-
logists, immunologists, physiologists, biochemists, physical chemists, nuclear scientists and others—have all contributed to the advancement of knowledge. The physician of today has at his disposal potent investigational tools for diagnosing the presence, the severity and the pathogenesis of vitamin B₁₂ deficiency. He also has available an abundant supply of the vitamin, in pure form at relatively low cost, for the treatment of patients with established deficiency.

These great advances should not, however, blind us to our ever greater ignorance still existing in many of the areas dealt with in this review. The profitable interdisciplinary cooperation of the past needs to be continued and extended if the bounds of our ignorance are to be further decreased.

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Author’s address: S. J. BAKER, Wellcome Reseach Unit, Christian Medical College Hospital, Vellore, Madras State (India).