REVIEW ARTICLE

A NEW CONCEPT OF THE PHYSIOLOGICAL ROLE OF VITAMIN B_{12}

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THE isolation of vitamin B_{12} from liver or fermentation broth sources has always involved one important and apparently inevitable step, namely, the separation of the vitamin from associated protein-like material. The nature of the binding is unknown. That it involves more than physical admixture has been evident since the early attempts to crystallize the vitamin were continually thwarted by the presence of protein-like impurity. Thus Lester Smith and Cuthbertson in 1948[1] had difficulty in freeing the vitamin from bound material in purified concentrates from liver. These workers identified some of the amino acids in the complexes of vitamins B_{12} and B_{12b} but, after the crystallization of the pure vitamin, they do not appear to have pursued the nature of the natural binding in liver extracts any further.

The effectiveness of crude liver preparations when given by mouth in the treatment of pernicious (Addisonian) anaemia has long been known. Recently Robinson and others[2] have shown most of the vitamin B_{12} in crude liver extracts to be bound; the material is not bound to protein since the bound vitamin can be dialysed through Cellophane. The possibility of a complex formation with a peptide of low molecular weight was not excluded. In contrast to crude liver preparations the crystalline vitamin is ineffective, even in large doses, in the oral therapy of pernicious anaemia. After its isolation it seemed for a time that the vitamin and the growth factor present in animal proteins, and necessary for supplementing the vegetable protein diets of animals, might prove to be identical. Later it became evident that this was not so.

These and other considerations led the authors to believe that for both animal growth and pernicious anaemia a vitamin B_{12}—peptide complex was the active substance since it was more readily absorbed and more nutritionally acceptable than the uncombined vitamin. Such a complex could be either ingested, or synthesized in the blood stream by the combination of the injected vitamin and available low molecular weight protein or peptide at blood pH, and also might not require a high degree of specificity in the nature of the bound material.

The generally accepted theory of action of vitamin B_{12} in oral therapy is that of Castle who postulated that the vitamin must be bound to some protein-like material which is derived from the alimentary tract and which facilitates absorption of the vitamin. Ternberg and Eakin[3] were the first to demonstrate that the vitamin combined with a principle in normal human gastric juice to form a non-dialysable complex. The vitamin in

this combination was not available to micro-organisms that need it as a growth factor which implies that the vitamin was binding preferentially to high molecular weight material. Glass, however, has recognised that the transport of a large protein molecule across the gut wall necessitates introducing some additional factor ("acceptor factor"). If Ternberg and Eakin's suggestion, that the vitamin B_{12}-binding principle in gastric juice is identical with intrinsic factor and is non-dialysable, were so, then low molecular weight proteins or peptides would be entirely inadequate to effect absorption of the vitamin. But the inconsistent results which have been obtained with "intrinsic factors" have challenged the validity of this suggestion. It also does not appear to agree with the accepted principles of the digestion process, which is normally concerned with the provision of relatively small molecules for absorption. Thus molecular size might be an important criterion for absorption of the vitamin in a utilizable form.

Species specificity has been observed[4,5], for instance rat gastric juice has intrinsic factor activity (i.e., results in the absorption of oral cyanocobalamin) for the rat only. But, gastric juice is such a complex mixture that molecular size could still be a more important consideration than species specificity. If this should be so then vitamin B_{12}-peptide complexes released from mould or bacterial cells might be as active clinically as those released from liver cells. Thus, instead of isolating the pure vitamin from liver or fermentation liquors, and combining it with animal intrinsic factors, it should be possible to isolate the vitamin in a natural, combined state with improved absorption characteristics and therefore more effective orally than the pure vitamin.

During the past few years this possibility has been investigated in these laboratories. Encouraging clinical results have confirmed the oral effectiveness of vitamin B_{12}-peptide complexes prepared in the laboratory from fermentation sources[117]. The present article reviews the theoretical aspects on which the oral therapy of pernicious anaemia has been based. It endeavours to show the inconsistencies and contradictions inherent in the currently accepted approach to this problem. The new approach, which already has appreciable clinical support, is here correlated with the wider aspects of the role of vitamin B_{12} in animal nutrition.

Aetiology of "Addisonian" Pernicious Anaemia

Since the early nineteenth century it has been believed that pernicious anaemia is associated with imperfect assimilation of nourishment. Fenwick in 1877 and 1880 demonstrated that the digestive power was absent from an extract of the gastric mucosa of a patient who had died from pernicious anaemia. That achlorhydria accompanied pernicious anaemia was established by Cahn and von Mehring in 1886[6] and Faber and Bloch in 1900[7]. Lazarus in 1898 considered that the megaloblastic state of the bone marrow might be due to an arrest of maturation. These early observations were overlooked until 1926 when Minot and Murphy[8] demonstrated that liver administered orally could effect remission in pernicious anaemia. On the basis of a series of well known studies

(for a review see Castle[9]), Castle in 1929 put forward his classical hypothesis which linked the loss of the haemopoietically active principle in liver with the underlying gastric defect. Essentially it was established that normal human gastric juice contained a factor, the "intrinsic factor", which when given orally together with an "extrinsic factor", found in meat, was able to cause haematologic remission in pernicious anaemia. Castle and Minot in 1936[10] assumed that the gastric (intrinsic) factor reacted with the food (extrinsic) factor to produce the haematologically active principle, which was stored in the liver, kidney and other tissues. Since the discovery of vitamin B_{12} by Folkers and colleagues and by Lester Smith in 1948 and the demonstration that the crystalline vitamin caused remission in pernicious anaemia, *either* when injected parenterally or when given orally together with gastric juice, it has been thought that the role of intrinsic factor was to promote the intestinal absorption of the vitamin in a specific manner[11]. That is to say, vitamin B_{12} is considered to be the extrinsic factor. But according to Glass and others[12], the intestinal absorption of the vitamin—even in the presence of normal amounts of intrinsic factor—may be surprisingly small. Thus an increase in the amount of ingested vitamin from 0·5 to 50 μg. results only in an increase in hepatic accumulation from 0·45 to 1·5 μg. These authors, therefore postulated an intramural "intestinal acceptor of the vitamin" and the failure of increasing doses to be absorbed could be explained by the saturation of this acceptor. The limited capacity for absorption of the vitamin even in the presence of intrinsic factor, may explain some of the difficulties in treating sufferers from pernicious anaemia by the oral route.

According to present concepts (see e.g., Nutrition Reviews, 1955) "the absorption of vitamin B_{12} by the human intestine is a complicated problem involving the presence of a facilitating substance (intrinsic factor) and possibly an intestinal acceptor substance". Thus, if vitamin B_{12} is the extrinsic factor, then two additional factors must be involved in its transfer across the intestinal mucosa of a normal individual.

Before passing on to the consideration of the role of intrinsic factor in pernicious anaemia, some clinical results which have been obtained with oral crystalline vitamin B_{12} alone will first be considered.

Oral Absorption of Vitamin B_{12} Without a Source of "Intrinsic Factor"

When crystalline vitamin B_{12} became available it was tested orally and found to be relatively ineffective. However, when given with normal gastric juice, it proved to be nearly as effective as by injection[11]. This led to the idea that extrinsic factor and the vitamin are the same.

Ungley[13] has summarized the results of several workers. He concluded that daily oral doses of 5 or 10 μg. of the crystalline vitamin (21 cases) either had no detectable effect, or more often, gave a trivial response similar to that produced by a single injection of 1 or 2 μg. With daily doses of from 25 to 450 μg. (27 cases) results were extremely variable. With few exceptions absorption appears to have been less than one per

cent. Large doses of 3,000 μg. were effective even without a source of intrinsic factor according to Ungley[14] and in 21 patients this dose was equivalent to an injection of 40 μg.

Reisner and colleagues[15] found that it was possible to maintain 43 persons with pernicious anaemia for periods of 12 to 28 months on doses of 1,000 μg. at weekly or two-weekly intervals. Chalmers[16], having previously shown that large doses of the crystalline vitamin were effective orally[17], studied a further 14 patients with pernicious anaemia in relapse. With daily oral doses of 50 to 200 μg. of the vitamin first thing each morning when fasting, results were good in studies of up to 2 years and most patients obtained complete remission with a return of serum vitamin levels to within normal range (200–1,000 μμg./ml.). Estren and Wasserman[18] claimed maximal responses in three of nine patients, sub-maximal in five and one failure at a dosage of "5–16·8 μg. of vitamin B_{12} . . . one to three times daily". The degree of response in the sub-maximal cases is difficult to assess as details of progress and duration of treatment are shown in two cases only.

Glass, Goldbloom and Boyd[19], using the method of hepatic uptake of radioactive vitamin B_{12} found that people of over 60 years with gastric hypo- or anacidity had a significantly lower intestinal absorption of the vitamin than those of the same age with normal or hyper-acidic gastric secretion. Glass, Pack, Mersheimer, Kusnick and Laughton[20] had likewise previously shown that patients with total gastrectomy, who frequently develop pernicious anaemia, observed no hepatic uptake of the radioactive crystalline vitamin after oral administration. The defect could be corrected by adding a source of "intrinsic factor". This reduced absorption of the crystalline vitamin is in agreement with the incidence of incipient megaloblastic erythropoiesis found in such elderly achlorhydric patients despite the absence of manifest symptoms of pernicious anaemia[21].

The oral absorption of the crystalline vitamin is appreciably greater in the normal subject than in the patient with pernicious anaemia. Using the technique of Welch and Nichol[22], which consists in giving orally a dose of 0·5 μg. of radioactive vitamin B_{12} to a fasting subject and then measuring the radioactivity of all the stools passed during the next 5–6 days, Callender Turnbull and Wakisaka[23] found that in ten subjects with normal haematological findings a mean of 31 per cent of the radioactivity was present in the faeces. In 13 patients with pernicious anaemia a mean of 89 per cent was recovered. In general it can be stated that, while patients showing a poor absorption by this diagnostic test are not necessarily suffering from pernicious anaemia, those with the disease normally show a low absorption of the crystalline vitamin.

Intrinsic Factor

Castle's hypothesis led inevitably to an enormous amount of work in the search for the intrinsic factor. Since the discovery of crystalline vitamin B_{12} in 1948, Castle has modified his original theory in so far as he has equated the vitamin with the extrinsic (or food) factor. Extensive reviews

on the subject of intrinsic factor have been made by Ungley[13] and more recently by Gräsbeck[24] and the following summary serves to indicate the present confused state of knowledge.

In the pig, intrinsic factor is not only present in the gastric juice[25], but also in the pure duodenal juice[26]. According to Ungley, preparations of every portion of the gastric intestinal tract of the pig (except the fundus (corpus) of the stomach) are haemopoietically effective when administered with vitamin B_{12}. Experimental work has been further complicated by the presence of both intrinsic and extrinsic factors[27,28] in the stomach and intestine of the normal animal. Even normal human gastric juice apparently contains the vitamin[29].

In man, the intrinsic factor is found in gastric juice but not in saliva and probably not in pure duodenal juice[30]. Likewise Schilling and others[31] have demonstrated, that the stomach is the only site in man for the production of intrinsic factor. Moreover, in contrast with the pig, it is the fundus (or corpus) and the cardiac end which are active, not the pyloric region[32–33]. Thus pernicious anaemia is found together with characteristic atrophy of the fundus region[34–36]. Histological evidence shows that the atrophic changes in the stomach persist during remission.

Properties of Intrinsic Factor

The properties of "intrinsic factor" from gastric juice have never been clearly defined and its behaviour has varied according to the stage of fractionation and the composition of the medium[13]. Ungley has pointed out that it is even more difficult to gauge the properties of intrinsic factor derived from preparations of stomach and intestine where, besides being associated with numerous impurities, it may be bound to the vitamin. He has concluded that "intrinsic factor" is unstable and that attempts at concentration usually involve considerable loss of activity.

Thus Seitz filtration, at acid pH, of normal gastric juice apparently led to considerable loss of activity in one instance[37] but Seitz filtered, neutral gastric juice was found active for potentiating the megaloblast-ripening effect of the vitamin in marrow culture[38]. The haemopoietic activity of an acid (pH 4·6) extract of stomach was destroyed by heating at 60 to 70° for 30 minutes[39]. Intrinsic factor in pyloric mucosa extract and in gastric juice was not destroyed by alkalinity at pH 9·8 for 30 minutes at room temperature.

Although intrinsic factor in gastric juice is said to be inactivated by 70 per cent ethanol[40], Wilkinson and Klein[41] used 92 per cent ethanol to precipitate a haematopoietically active material from stomach press juice. Prusoff and others[42] found that most of the "intrinsic factor" activity of desiccated stomach was in the fraction precipitated by 35 to 55 per cent saturated ammonium sulphate.

It is generally agreed that intrinsic factor will not pass through a semipermeable membrane such as Cellophane[43]. Helmer and Fouts[44] recommended ultrafiltration of gastric juice as a preliminary to concentration. Bethell and colleagues[28] used dialysis to remove the vitamin from

extracts of duodenal mucosa. Goldhamer and Kyer[45] also found that intrinsic factor was not dialysable.

It was found that the glandular mucoprotein fraction of gastric juice potentiated to some degree the effect of orally administered vitamin B_{12}[46] and the mucoprotein nature of intrinsic factor has since become widely accepted.

Hall, Morgan and Campbell[47] demonstrated that the intrinsic factor of gastric juice is effective even when given 2 hours after the vitamin. Even before the vitamin was discovered, Castle and Ham[27] found that when beef was given as long as 6 hours before gastric juice there was still a haemopoietic response. The daily oral administration of buffered mixtures of gastric juice and beef at pH 1·8 or 2·5 did not lead to the increased blood production observed when such mixtures were given at pH 5 or 7[48,49]. At the time this was thought to mean that the high acidity was preventing an essential combination between "intrinsic" and "extrinsic" factors which could take place only *in vivo* and which presumably occurred in the upper part of the small intestine. The finding of Callender and Lajtha[38] that the vitamin is inactive in bone marrow culture unless potentiated by gastric juice suggests that "the vitamin B_{12}—intrinsic factor complex may be the active form of the vitamin"[13].

Vitamin B_{12}—Binding Principle

Ternberg and Eakin[3] were the first to demonstrate that normal human gastric juice as well as hog stomach preparations contained a principle "apoerythein" which combined stoichiometrically with crystalline vitamin B_{12} to form a complex which was nondialysable and non-dissociable on dialysis. The vitamin in this complex was not available to micro-organisms (*E. coli*, *L. leichmannii* and *L. lactis* Dorner) that need it as a growth factor, a property which can be used for the assay of the binding principle. Heated gastric juice lost its capacity to bind the vitamin and heat released the latter from the complex. Eakin (cited by Hall[50]) found the concentration of apoerythein to be low in the gastric juice of pernicious anaemia patients and this led to the suggestion that apoerythein was identical with Castle's intrinsic factor or a component of it.

Several methods for assaying the binding capacity of the vitamin were subsequently developed—all based upon one of the following principles outlined by Ungley[14].

(i) Only the unbound vitamin can be utilised by micro-organisms. (Microbial Growth Inhibition technique). (ii) Only the free vitamin is dialysable. (iii) Only the free vitamin can be adsorbed on to certain micro-organisms (Microbial Adsorption Inhibition). (iv) Free and bound vitamin behave differently on electrophoresis. Most, if not all, of the work on the preparation of intrinsic factors, has been based on these principles. Much depends, however, upon what is meant by the arbitrary terms "free" and "bound" and these terms are discussed later.

Latner and others[51–53] claimed the isolation in a chemically pure form of the "intrinsic factor" which from ultracentrifugal studies had a molecular weight of about 15,000.

Glass[54], however, has disputed this, and has criticized the method (faecal excretion of radioactive vitamin) used for the measurement of intrinsic factor activity. More recently, Thompson and Latner[55] have claimed a preparation that was able to produce a haematopoietic response when given in a 4 mg. dose together with 5 μg. of the vitamin daily for ten days.

Hog bile is rich in a heat-stable vitamin binder[56]. Human duodenal juice has been reported to bind the vitamin[57], but to have little or no intrinsic factor activity[30]. The latter authors demonstrated that saliva did not possess intrinsic factor activity, yet Beerstecher and Altgelt[58] showed that it contained apoerythein. It is by no means certain, however, that apoerythein is identical with intrinsic factor. Doubt has been cast on the validity of deductions based on activity being associated with vitamin B_{12}-binding power, because the vitamin binds itself to many substances without "intrinsic factor" activity. This applies to egg-white lysozyme, sow's milk protein[59] and even to some fractions from pig's duodenum[50]. Wijmenga's[60] purified cobalamin protein is another example.

Purified Intrinsic Factor Preparations and their Activity

Prusoff, Welch, Heinle and Meacham[61] further purified an intrinsic factor preparation, "Ventriculin", from hog stomach employing saline extraction and ammonium sulphate fractionation. The fractions were tested both clinically and for binding capacity by the dialysis and microbial growth inhibition methods. No correlation was found of the ability to bind the vitamin and the clinical "intrinsic factor"; the fractions with the lowest vitamin-binding power had the greatest clinical effect! Again, Everse, Lens and Wijmenga[62] report that the clinical "intrinsic factor" effect of fractions of human gastric juice and of hog stomach does not correlate with the vitamin B_{12}-binding power measured by the microbial growth inhibition technique. Further, though most patients with pernicious anaemia lack vitamin B_{12}-binding capacity in their gastric juice, occasional patients have been found to possess this activity[63–64]. However, presumably the lack of "intrinsic factor" in gastric juice, need not be absolute[65]. Again, Virtanen and Tanksanen[66] isolated a substance from calf stomach which had no vitamin B_{12}-binding power but still evoked some haematologic response.

Wijmenga, Thompson, Stern and O'Connell[67] added the crystalline vitamin to a clinically active hog stomach preparation and isolated a pure conjugated cyanocobalamin protein, the protein part of which they considered to be identical with the vitamin-binding factor in hog gastric mucosa (and probably in human gastric juice also). Electrophoresis, alcohol fractionation and ammonium sulphate fractionation gave two red coloured products with molecular weights of 128,000 and 100,000 respectively. Later[60], these authors purified the cobalamin protein further and claimed that the vitamin content was as high as 18·5 μg./mg. Assuming that one molecule of vitamin is bound per molecule of protein the molecular weight is 70,000. The purified complex, however, failed

to show any clinical intrinsic factor activity after daily oral dose administration of amounts corresponding to 5 μg. of bound vitamin.

In the Lederle Laboratories, Williams, Ellenbogen and Esposito[68] prepared a complex which was haematopoietically active in a daily dose of 1–2 mg. The complex was prepared by means of alcohol and salt solution precipitations and by enzymic digestion. An end-product was obtained by dialysis and ultra-filtration which was known as the ultra filtration residue (U.F.R.) This consisted of two components, a low molecular weight component (about 5,000) comprising 70 per cent of the material and a heterogeneous high molecular weight component (of 100,000 and 500,000) comprising the remaining protein. Incomplete clinical trials showed that the lower M.W. component had the more intrinsic factor activity. The vitamin B_{12}-binding capacity of the U.F.R. fraction was 220 μg./g. which would mean that, if all binding capactiy were to be ascribed to the lower M.W. component, 1,000 molecules would be necessary for the binding of one vitamin molecule. From this and other considerations, the authors concluded that vitamin-binding is not a property of "intrinsic factor". The components of U.F.R. were mucoproteins or mucopolypeptides.

In contrast with these experiments which indicate that "intrinsic factor" activity is a phenomenon different from vitamin B_{12}-binding capacity, Hoff-Jorgensen and Landboe-Christensen[69] and Noer[70] compared the anatomic localization in the hog stomach of the vitamin-binding factor (determined by the microbial adsorption technique) and intrinsic factor activity and found the localization to be the same. The microbial adsorption inhibition technique is based on a concept of Burkholder[71] who showed that concentrates of hog stomach were able to prevent bacterial uptake of the vitamin by binding it. He found that vitamin-binding paralleled the clinical "intrinsic factor" activity of his preparations and suggested that "intrinsic factor" acted by inhibiting the uptake of the vitamin by the intestinal flora. The strain of *E. coli* which the Danish team used was one which had been isolated by Hoff-Jorgensen, Skouby and Gad Andresen[72] from the faeces of a patient with pernicious anaemia. Their later studies showed that the vitamin-binding capacity in the human stomach is strongest in the fundus[73] which is the principal site of "intrinsic factor" activity in man according to Fox and Castle[32].

In 1954 Gad Andresen reported the isolation, from a commercial intrinsic factor preparation (Bendogen R), of the vitamin B_{12}-binding factor in a pure form and believed the cobalamin protein to be identical with that isolated by Wijmenga and his associates. In contrast to the latter substance, however, the complex caused complete haematological remission when given in a daily dose of 1·2 mg. corresponding to 10 μg. of bound vitamin.

Absorption studies by Baker and Mollin[74], using radioactive vitamin B_{12}, indicated that the reaction between it and intrinsic factor is a stoichiometric one, which would argue in favour of the identity of "intrinsic factor" and the binding principle, since Ternberg and Eakin[3] found the binding by "apoerythein" to be more or less stoichiometric. However,

earlier studies[75,76] than those of Baker and Mollin had indicated that the reaction between "intrinsic factor" and the vitamin is not stoichiometric. Raine[77] found that the pure intrinsic factor of Latner and co-workers bound vitamin B_{12} in relation to the vitamin concentration and not stoichiometrically. Thus the question of whether "intrinsic factor" and the vitamin B_{12}-binding principle are identical is not yet settled.

Commercial "Intrinsic Factor" Preparations

In clinically healthy individuals, intrinsic factor preparations were extremely variable in their effects[78]. Nevertheless, satisfactory treatment of pernicious anaemia by administration of crude preparations of hog's stomach has been achieved for years. Thus, Wilkinson[79] reported that of 441 patients treated for six or more years with desiccated hog's stomach, only fifteen relapsed through neglect of treatment and they responded again promptly when treatment was given. Although the heterologous nature of "intrinsic factor" preparations has often been blamed as a possible cause of their failure—and the latest results by Schwartz, Lous and Meulengracht[80] of Copenhagen would seem to support such a view—this can hardly be a serious factor. The belief has been expressed (*Lancet annotation*, 1957, **1**, 775) that the cause of the failure of commercial preparations might lie in their method of preparation as well as in any possible resistance or inhibition arising because of the heterologous source of "intrinsic factor". This editorial view point is in line with the belief expressed later in this article that the majority of even the successful preparations which are being made at present are probably effective more by fortuitous contamination with low molecular weight material of an active nature than by their own essential nature. Hence if the mode of preparation is at fault, a new approach to the problem would seem to be necessary. However, before discussing this there are other relevant topics to be considered; one of these concerns the arbitrary terms "bound" and "free" vitamin B_{12}.

"Bound" and "Free" Vitamin B_{12}

Discussions of the oral absorption of vitamin B_{12} assume that the "free" vitamin in animal foods is available for combination with an intrinsic factor to form the anti-pernicious anaemia principle. That is to say, it is considered that the "free" vitamin in such foods is identical with Castle's "extrinsic factor". It is doubtful, however, if much vitamin B_{12} is "free" at all, judging by the extraction procedures which are necessary to release it from tissues and foods for microbiological assay. Even in blood serum it mostly occurs "bound" but it can be converted to the "free" form by heating at 100° in acid medium for 15 minutes. It cannot be emphasized too strongly, that these terms are quite arbitrary. "Free" vitamin B_{12} is by no means synonymous with pure uncombined cyanocobalamin; the latter does not seem to occur naturally. The term "free" is generally taken to mean assayable by micro-organisms and, presumably, this means that some, undefined, form of the vitamin is capable of diffusing through the cell walls of the assay organism. However, even when attached to

quite large molecules, the vitamin may still be assayed microbiologically with certain organisms and indeed the assay value given is specific for the vitamin part only. Even in mixtures such as blood sera and liver extracts the values for "free" vitamin B_{12} apparently correspond to the amount of pure uncombined vitamins present, when assayed against organisms like *Euglena gracilis* z strain[81] or *Ochromonas*. Other organisms such as *E. coli* give higher values[82] and this is taken to indicate a greater specificity for vitamin B_{12} by *Euglena* and *Ochromonas*. Certainly the vitamin content of these complex forms is more accurately determined by the latter organisms, but the higher values obtained with *E. coli* may be due to an additional stimulus to growth by the peptide portion of the so-called "free" vitamin.

Normal Absorption of Vitamin B_{12}

Since the vitamin in foods is normally bound to protein material which is unavailable to micro-organisms and requires a pre-digestion, it may be considered that in the animal also the vitamin or "extrinsic factor" is likewise unavailable without prior digestion. On Castle's theory (that the crystalline vitamin is the "extrinsic factor") this digestion would involve splitting the pure cyanocobalamin from the protein complex in order that it could then combine with intrinsic factor in the gastric juice. It would seem equally feasible, however, that partial digestion such as occurs in the release of "free" vitamin from "bound" vitamin in sera and foodstuffs could take place. Even if some pure cyanocobalamin were released to combine with intrinsic factor this would not exclude the likelihood of the larger amount of "free" vitamin B_{12} (i.e., vitamin bound to low M.W. peptide material of a diffusible nature) coming from the food and entering the portal blood stream in that form. Thus neither "extrinsic factor" nor "intrinsic factor" would have a separate existence but would both be combined as a vitamin B_{12} peptide complex. This would argue against a specific "intrinsic factor" and, if such a hypothesis were true, then non-specific peptide complexes might be active orally in persons with pernicious anaemia.

Extraction of "Free" Vitamin B_{12} from Fermented Broths and Foods

In the normal extraction from fermented broths, an acid treatment at low pH values (e.g., pH 2·0 for 1 hour) or a heat treatment as used by Smith and Ball[83] is necessary to release all the vitamin from the cells in a "free" assayable state. When the vitamin is "free", i.e., diffusible, it will assay with some organisms as does pure cyanocobalamin. The vitamin must be combined in some way with protein-like materials within the cell since it does not diffuse into the medium to any appreciable extent during fermentation.

It is interesting to speculate on how this "freeing" of the "bound" vitamin occurs since it may have an analogy in human (or animal) nutrition. It could be that the vitamin B_{12}-protein complex of high molecular weight and undetectable on assay, is hydrolysed by the acid at some intermediate peptide bond(s) or is broken down from a polymer to a monomer, the vitamin remaining attached to its neighbouring peptide

portion. This type of extraction/hydrolysis might occur in the normal stomach which, of course, contains acid. The food is usually less acid than pH 2 but there would be a longer time of digestion and an augmenting effect of the enzymes of proteolysis. It is of interest to note that although achlorhydric patients do not necessarily become anaemic in the "pernicious" sense, according to Schilling and others[31] they absorb less of the vitamin than normal subjects.

The failure of pernicious anaemia patients to make use of vitamin B_{12} produced in the large intestine (colon) from which absorption of (diffusible) materials is known to occur normally, is probably due also to the vitamin being retained within the micro-organism which will normally be excreted in the faeces long before autolysis, thus preventing the patient from utilizing the vitamin.

The Role of Vitamin B_{12} in Nutrition

Animal Nutrition

Attempts in America to eliminate scarce and expensive animal proteins from the diets of farm animals led to the appearance of symptoms of nutritional deficiency. The growth rates of chicks and pigs were adversely affected and hens produced eggs of low hatching potentiality. Raising the level of certain sources of vegetable protein, such as that from soya bean, worsened the position. The deficiency signs could be prevented or cured by the addition of fish-meal or cow manure[84,85]. It was later shown that the factors in cow manure concentrate and fish solubles had very similar properties[86]. In 1946 Cary and others[87] demonstrated that liver extracts which also contained a rat growth factor were effective in pernicious anaemia. About the same time Zucker and Zucker[88,89] discovered a rat growth factor "zoopherin" in animal proteins. Thus it appeared likely that the factors were identical. That synthesis by micro-organisms seemed to be involved was suggested by the activity of cow manure and this was further supported by the observation that a factor was produced by the fermentation of food materials in chick faeces during warm weather, since chicks could manage without animal protein in their food at that time. Stokstad and others[90] confirmed the identity of the animal protein and anti-pernicious anaemia factors, when they showed that an organism isolated from hen faeces could synthesize a factor effective in promoting chick growth and in treating pernicious anaemia in man.

When crystalline vitamin B_{12} became available it was claimed that given orally or by injection, it had the same effect for chicks as the animal-protein factor[91,92]. Subsequent work, showed that the crystalline vitamin together with vegetable protein cannot completely replace animal protein from natural sources. Thus, Stokstad, Jukes, Pierce, Page and Franklin[93] found that a concentrate derived from fermentation liquors of *Streptomyces aureofaciens* gave a greater growth response than crystalline vitamin B_{12}. They concluded that chicks require an unidentified factor in addition to the vitamin. Ershoff[94] fed immature rats massive doses of thyroid together with a purified ration containing casein as the dietary protein. A marked retardation occurred both in the body and gonadal

weight which was completely counteracted by administration of liver residues. Crystalline vitamin B_{12} was without effect.

Heuser and Norris showed in 1944 that growth was retarded in chicks not receiving animal protein foods. After the discovery of vitamin B_{12} it was shown that soybean cereal rations are deficient in the vitamin and the increased growth effect of animal proteins was thought to be due to the vitamin. But the work of Heuser and Norris[95] showed that this could not be so since, even with the addition of more vitamin to vegetable protein feeds than was added to animal protein feeds, there was a greater growth increase with the animal protein feeds. There was also a greater growth with vegetable foods and antibiotics, possibly due to the latter stimulating the intestinal flora to synthesize animal protein factor or to the presence of A.P.F. in the antibiotic concentrate. Also Briggs and Beeson[96] showed that a combination of vitamin B_{12} and streptomycin potentiated growth. This same augmenting effect of antibiotics in the presence of the vitamin has been confirmed with both penicillin and aureomycin[97].

In rats, Sherman, Schilt and Shaefer[98] showed that 3 per cent fish solubles gave double the increase in growth when compared with that produced by optimum levels of the vitamin. This magnitude of response must result from unidentified factors in fish solubles. It is interesting to note that antibiotics were not sources of unidentified factors.

Moulds and bacteria have been grown on solid or semi-solid vegetable media to produce animal protein factor to replace fish meal, fish solubles and liver extract[99,100]. These organisms produced vitamin B_{12} and other factors. It is well-known that antibiotics alter the intestinal flora and that such an alteration in animals might account for the production and utilization of additional growth factors. It is conceivable that some of these additional growth factors might be more readily absorbable forms of vitamin B_{12}, i.e., forms in which the material bound to the vitamin is of low molecular weight (peptide). That the form of the vitamin is important is shown also by the work of Coates and others[101] who found that the vitamin in sow's milk is only poorly utilized by the chick but is readily available to the piglet.

Human Nutrition

After the therapeutic value of the vitamin had been established its ability to improve partially the biological value of vegetable proteins was demonstrated in various animal species. But there has, however, been a delay in obtaining proof of its "animal protein factor" activity in human nutrition. A distinct growth promoting effect has been claimed when given as a dietary supplement to children in growth failure[102] which has not been confirmed[103,104]. Nevertheless, human dietary deficiency of the vitamin, that is a clinical condition apparently not due to an absence of "intrinsic factor", has recently been described among persons in Britain living entirely on vegetable foods[105,106].

A megaloblastic anaemia in which nutrition plays at least some part is reported by Foy and others in East Africa. In their latest paper they

show that those who respond to treatment either with penicillin or with relatively small oral doses (80 μg.) of vitamin, have low serum levels (20–100 $\mu\mu$g./ml.) of the vitamin. The diet of these people is poor in animal protein and rich in carbohydrates and gastric function is usually unimpaired. There would seem to be two possible explanations of this phenomenon. Firstly it may be that penicillin encourages the growth, in the intestine, of organisms which produce vitamin B_{12} in sufficient quantities to overcome the nutritional deficiency. Alternatively this diet may encourage the growth of organisms that compete for the available vitamin, and these organisms are presumably sensitive to penicillin.

In food, vitamin B_{12} is usually associated with animal protein; it has been detected in only one or two vegetable foods, such as groundnuts and, if any purely dietary deficiency ever occurs it is usually among vegetarians. Wokes, Badenoch and Sinclair[105–109] have investigated vegans who eat no animal protein at all not even dairy products; and groundnuts are often absent from their diets. Even so some of these people seem to escape deficiency symptoms for as long as five years on the diet. The commonest and earliest symptoms are in the mouth, a sore tongue being often prevalent. In a group of 150 vegans, 27 per cent had oral symptoms and paraesthesiae developed in 20 per cent. Amenorrhoea and other menstrual disturbances were common and appeared early in women aged between fifteen and forty-five years. Serum B_{12} levels were also low 45–193 $\mu\mu$g./ml. compared with the value (200–320 $\mu\mu$g./ml.) obtained for the controls. Haemoglobin levels were normal but red cell counts in some were a little low. Wokes and others point out that vegetables contain comparatively large amounts of folic acid; and since folic acid is known to precipitate the onset of neurological symptoms in classical pernicious anaemia, it is conceivable that in the diet it may have a similar effect on vegans who have become deficient in vitamin B_{12}.

Wokes[108] reported further data on children and stated that vitamin B_{12} requirements become more critical after early weaning, when dietary supplementation with vitamin B_{12} may be more effective. Wokes considers that possibly the reason why so many people in the Middle and Far East living on diets low in protein do not develop symptoms of vitamin B_{12} deficiency is due to their widespread use of fermented and germinated food. In vitamin B_{12}-deficiency in vegans there can be no shortage of "intrinsic factor" because the symptoms are relived by the administration of animal protein such as milk, which is a good source of the vitamin.

Further evidence that normal digestion is the only factor missing in pernicious anaemia comes from the work of Bonsdorff and his colleagues on fish tape-worm anaemia. This worm resides in the lower part of the intestine and has been shown[110] to be a rich source of cyanocobalamin. The dried worm alone is effective in curing the megaloblastic tape-worm anaemia and together with normal gastric juice it is effective in Addisonian pernicious anaemia. These experiments show that in the normal human intestine the vitamin must be in a readily assimilable form in order to diffuse readily through the body of the tape-worm.

In commenting upon both animal protein factor in animals and upon vitamin B_{12} deficiency in humans, it only remains to be pointed out that if a diffusible peptide complex of the vitamin were the truly requisite factor in animal nutrition, many if not all of the discrepancies could be more easily explained. Certainly the production by micro-organisms, either in the animal directly or via animal protein food, of bound forms of the vitamin which in the normal animal could be broken down to a more assimilable form, though still as a vitamin complex, is a feasible explanation both of the effectiveness of antibiotics and for the inadequate effect of the crystalline vitamin.

The New Approach

The theories of absorption of vitamin B_{12} have been critically examined. A new theory[117] is now put forward which is based on the assumption that a vitamin B_{12}-peptide(s) complex, and not the crystalline vitamin, is the active anti-pernicious anaemia principle. There is evidence for this in the poor absorption of pure uncombined cyanocobalamin given by mouth to pernicious anaemia patients, in the non-maturation of megaloblasts *in vitro* by the crystalline vitamin and in the effectiveness of oral liver therapy without added intrinsic factor. Murphy[111] has cautioned that "further critical study is necessary before vitamin B_{12} is accepted as a complete substitute for either whole liver or liver extracts" and it is significant that more vitamin is excreted when injected as cyanocobalamin than when injected as a purified liver extract (cobalamin-peptide).

In most foodstuffs, the vitamin occurs in a form which is non-assimilable unless it is broken down by proteolysis. Indeed the analysis of the "free" vitamin in foodstuffs involves appreciable preliminary hydrolytic procedures. In the normal human subject a preliminary digestion of protein occurs in the stomach at acid pH. This could result in the release of the pure vitamin from its protein combination, when it could recombine with protein degradation products of low molecular weight in the intestine. Alternatively, and the more likely, the vitamin may remain attached to protein while the latter is degraded to a point at which it could be either directly absorbed or further broken down in the intestine and then finally absorbed into the portal system, through the cellular wall of the intestine.

On such a theory it would be reasonable to expect the activity of combined forms of the vitamin given by mouth where the peptide portion is not homologous. Such results have indeed been obtained with both crude[112,113] and with purified[114] heterologous intrinsic factor preparations. The numerous failures might well be attributable to binding with protein of incorrect molecular size.

It is hard to conceive that, if for oral absorption a high degree of specificity is required in the protein part of the active complex, this specificity is likely to be achieved when cyanocobalamin is injected into the blood where at the pH of blood ready combination is possible with a wide variety of related materials of differing molecular weight. Specificity for particular amino acids is not unreasonable, however.

That vitamin B_{12} alone is insufficient to mature megaloblasts *in vitro*, whilst with normal gastric juice it does, indicates that some form of complex is essential. The true nature is unknown but, it would seem likely that the complex must diffuse as such into the portal blood stream through the gut wall and thence travel to the liver. From the liver it is likely that it is released again into the blood stream by the enzyme, erythropoietin[115], and that it then diffuses from the blood into the cells of the bone marrow to take part in haematopoiesis.

Some of the inconsistencies in intrinsic factor preparations may be partly explained by the variations in molecular size of the bound protein components in such mixtures. Thus vitamin B_{12}, if bound to high molecular weight material, even though the latter be comparatively pure, would not be expected to show oral activity in the patient with pernicious anaemia, e.g., the sow's milk protein complex of Gregory and Holdsworth[59] or the purified cobalamin protein (hog) of Wijmenga, Thompson, Stern and O'Connell[67]. These preparations had molecular weights of 55,000 and 70,000 respectively. Positive results obtained by using intrinsic factor preparations might be attributable to low molecular weight impurities, peptides and even amino acids being often extremely difficult to remove from some proteins according to Saidel[116].

Earlier work has led to attempts to prepare vitamin B_{12} complexes which have, as their protein component, a moiety of high molecular weight with non-diffusible properties. Good evidence for the new approach would be obtained if it could be shown that a non-specific bound form of the vitamin say one derived from a mould or bacterial source, were effective in promoting oral absorption of the vitamin (as the complex) in pernicious anaemia patients, particularly in maintaining them on small doses. Evidence that the vitamin is absorbed in a form similar to that in which it is ingested would be obtained if the vitamin B_{12}-peptide were to mature megaloblasts *in vitro*, a property which the crystalline vitamin does not possess.

If such a mould or bacterial B_{12}-peptide were effective as the anti-pernicious anaemia principle, it might seem reasonable to expect that it would be equally effective as the animal protein factor. For as the crystalline vitamin is not very effective *per se* in the oral therapy of pernicious anaemia, it is likewise not completely effective in replacing the animal protein factor in animal nutrition. However, it is well established that crude bacterial fermentations of the vitamin have animal protein factor activity and it would thus seem reasonable that a complex derived from these sources might be fully effective nutritionally for animals.

The above theory with special reference to pernicious anaemia, has already been tested experimentally and the results to date[117] fully support the novel and unorthodox approach to this problem.

A brief outline is given below of the isolation and properties of a vitamin B_{12}-peptide complex which possesses none of the properties which previously have been claimed to be necessary for absorption of the vitamin by the human intestinal tract.

Properties of the Complex

The active vitamin B_{12}-peptide complex (H.P.P./1) was derived initially from fermentation of a Streptomyces mutant under standard conditions. The cobalamin-peptide was released from the protein complexes within the cells and concentrated and purified by a series of steps involving ion exchange chromatography, counter-current solvent extraction, activated-carbon treatment and ammonium-sulphate precipitation. A red precipitate was finally obtained which possessed the following properties:

The vitamin B_{12} portion of the complex was estimated spectrophotometrically at 550 mμ, at which wavelength the interference of the peptide portion is very small. The ratio of peptide to vitamin B_{12} on a weight basis was 6·8 : 1 and, assuming a 1 : 1 molecular ratio of vitamin to peptide, the molecular weight of the complex would be about 10,000.

The true figure was probably less than this in view of the fact that the complex was dialysable and ultrafiltrable through Cellophane and collodion membranes. The complex did not appear to dialyse, however, against ammonium sulphate solution under conditions which enabled the uncombined vitamin to do so.

The ultra-violet and visible absorption spectrum of H.P.P./1 possessed absorption maxima at 277 mμ, 361 mμ, and 550 mμ—i.e., in the same positions as those of vitamin B_{12}. The maximum at about 277 mμ was appreciably greater than the corresponding peak value for the pure vitamin.

Both paper chromatography and paper electrophoresis showed the presence of only one vitamin B_{12}-peptide. Acid hydrolysis of the peptide component by 6N HCl at 100° for 24 hours yielded a mixture of amino acids, of which the following were identified by paper chromatographic separation and appropriate staining: glutamic acid, aspartic acid, glycine, alanine, valine, proline, serine, threonine, arginine, cystine (or cysteine), leucine, *iso*leucine, phenylalanine, lysine, and histidine. Methionine and hydroxyproline were not detected.

Alkaline hydrolysis, followed by paper chromatography, established, with specific reagents—e.g., Ehrlich's reagent—the presence of the essential amino acid tryptophane.

For accuracy in administration of the doses of active complex required, the vitamin content was estimated both spectrophotometrically and by direct microbiological assay using *Ochromonas malhamensis* as the test organism. That the vitamin in H.P.P./1 was directly assayable by organisms that require the vitamin as a growth factor shows that, according to generally accepted terminology, the vitamin was "free" and not "bound". The vitamin in H.P.P./1 also matured megaloblasts *in vitro*, a property not shared by the crystalline vitamin.

Preliminary Clinical Reports

Four males and two females, aged 42–72, with newly diagnosed pernicious anaemia were treated exclusively with H.P.P./1. A seventh case, which had begun to develop signs of subacute combined degeneration of the cord on treatment with another oral preparation containing intrinsic factor, is described separately.

Pernicious anaemia was diagnosed by an examination of peripheral blood, a histamine-alcohol test-meal, and biopsy of the sternal marrow (haemoglobin 100 per cent = 14·8 g./100 ml.).

Reticulocyte-counts were made for at least three days before treatment to exclude the possibility of a coincident spontaneous remission.

The treatment was carried out in two consecutive uninterrupted stages: (1) correction of the deficiency of vitamin B_{12}, and (2) maintenance of the patient on a lower dosage. Ultimately the success or failure of any preparation is judged on the second stage, for a good initial response to oral therapy is no guarantee of successful maintenance over long periods.

TABLE I

Case No.*	Duration of treatment (days)	Vitamin B_{12} (as peptide complex) (μg.)		Most recent count	
		Total dose	Average daily dose	Haemoglobin	Haematocrit
1	516	6,440	12·5	103	45
2	536	7,270	13·4	97	41
3	521	5,420	10·4	109	48
4	445	5,610	12·6	98	43
5	430	4,930	11·5	107	52

* The numbers here refer to those same cases reported in ref. 117.

Initial dose. As it is generally agreed that the liver contains 1–2 mg. of vitamin B_{12} in health, it was decided to give the first case 780 μg. of H.P.P./1 (vitamin B_{12} 100 μg.) daily for eight days, and then half that amount for fourteen more days (total dose of vitamin B_{12} 1,500 μg.). Dosage was varied slightly in subsequent cases but never exceeded this amount in the first three weeks' treatment.

Maintenance dose. As already mentioned Ungley[13] considered that a daily oral dose of 5–10 μg. of crystalline vitamin B_{12} had no detectable effect on the blood-picture. Thus, if a patient could be maintained in health within this range of dosage, it would be reasonable to presume that the preparation was effective. Accordingly 78 μg. of H.P.P./1 (vitamin B_{12} 10 μg.) was generally given as a daily maintenance dose.

Results

There was a good haematological and clinical response, which has been maintained in all six cases. A reticulocyte crisis with maxima of 11–17 per cent developed at about the end of the first week in every case, and the average daily increases in red cells and haemoglobin in the first 28 days' treatment were 47,000 per c.mm. and 1·07 per cent respectively. No case up to now has developed signs either of cord deterioration or of iron deficiency on treatment.

A preliminary report on six cases has already appeared[117] and the most recent results on these patients are summarized in Table I. Case No. 6, after 50 days treatment with the oral preparation, elected at her own request to have parenteral treatment "like her sister", who also suffered from pernicious anaemia and had been receiving injections for years. Up to the time of transference, her maintenance on oral therapy had been completely satisfactory.

It will be seen that 5 cases have now been maintained exclusively on H.P.P./1 for periods ranging from 430 to 536 days and that the average daily maintenance dose has never exceeded 13·4 μg. of vitamin B_{12}.

A further case, a woman, aged 52, with early subacute combined degeneration of the cord was treated with the oral peptide complex H.P.P./1.

For the first three weeks she received 2,340 μg. of H.P.P./1 (vitamin B_{12} 300 μg.) daily and thereafter 1,560 μg. of H.P.P./1 (vitamin B_{12} 200 μg.) daily for eight more weeks. Oral treatment then had to be abandoned owing to shortage of experimental material, and the patient was given 1,000 μg. of parenteral crystalline vitamin B_{12} weekly.

During the 11 weeks' oral therapy, however, an unequivocal subjective improvement took place both subjectively and objectively. During both oral and parenteral treatment, her blood-count remained essentially unaltered.

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