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## VITAMIN B<sub>12</sub>

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### The Discovery and Identification of Vitamin B<sub>12</sub>

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When the name vitamin B<sub>12</sub> was proposed by Rickes, Brink, Koniuszy, Wood & Folkers (1948), my colleagues and I debated whether we should adopt the term or retain the earlier name 'anti-pernicious anaemia factor', even when our red crystalline substance was shown to be identical with theirs (Smith & Parker 1948; Fantes, Page, Parker & Smith, 1949). It is now abundantly clear that this substance is indeed properly classified as a member of the B group of vitamins. Moreover, it seems very likely that vitamin B<sub>12</sub> might have been isolated several years earlier, had this fact been appreciated previously. We were looking not for a vitamin, but for a specific factor concerned in the treatment of pernicious anaemia. Accordingly, the only assay procedures that seemed worth serious consideration were those that attempted to induce in laboratory animals a condition akin to pernicious anaemia. Since this approach failed, in spite of many claims to the contrary, we had perforce to rely on the time-consuming (and inaccurate) clinical test to follow all fractionation procedures. Had we realized that we were looking for a vitamin, then I think we should have tried to develop at a much earlier date appropriate assay methods involving stimulation of growth in deficient animals or micro-organisms. The Americans were evidently less inhibited in their thinking and they did in fact devise a microbiological-assay method that must have been a great help in speeding up their fractionation procedures. It is a little ironical to recall that there are now at least a dozen assay methods for vitamin B<sub>12</sub>.

Other American workers were indeed well on the way towards the isolation of vitamin B<sub>12</sub> in the guise of the animal protein factor, using a chick-growth assay method.

It is not necessary to review again the early work on the purification of liver extracts. Our isolation of crystalline vitamin B<sub>12</sub> from crude liver extracts was only accomplished by the successive application of all, or nearly all, of the following steps: adsorption on activated charcoal followed by elution of impurities with 6 % phenol and of the active principle with 85 % phenol or hot 60 % alcohol; repetition of the adsorption and elution using less charcoal; precipitation of impurities with lead acetate; fractional salting-out with ammonium sulphate; extraction with *n*-butanol from solutions nearly saturated with ammonium sulphate, the active principle being re-extracted from the butanol with water; selective extraction with phenol or cresol mixed with several volumes of butanol or benzene; adsorption chromatography on

alumina from aqueous solutions; partition chromatography on damp silica using butanol or butanol-phenol mixtures as mobile phase; repetition of the partition chromatography; adsorption on a small amount of charcoal, followed by elution with 60 % propanol; precipitation with phosphotungstic acid; crystallization from aqueous acetone.

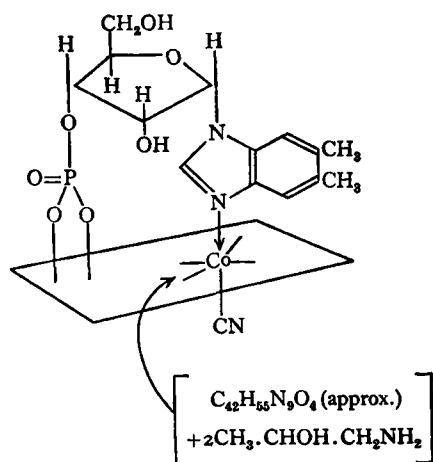
The yields in this multi-stage process were very poor. This can now be partly explained by the fact that we were dealing with a mixture of substances. Some of the initial activity was due to vitamin B<sub>12b</sub>, and more was doubtless converted into this form by loss of the cyano-group during the processing. The vitamin B<sub>12b</sub> would be separated from the vitamin B<sub>12</sub> and lost in some of the fractionation steps.

Vitamin B<sub>12</sub> is now manufactured by fermentation procedures similar to those used for antibiotics, the usual organism being *Streptomyces griseus*. The fermentation liquors respond to the same fractionation procedures as liver extracts. The following additional methods have mostly been gleaned from the patent literature: adsorption on fuller's earth and elution with solvent mixtures containing organic bases; adsorption chromatography on alumina from solutions in about 75 % methyl or ethyl alcohols; extraction of dry preparations with methanol or ethanol, followed by precipitation with excess of ether.

The procedure can be simplified if traces of cyanide are added from time to time to keep all the activity in the form of vitamin B<sub>12</sub> itself. For example, I have prepared crystalline radioactive vitamin B<sub>12</sub> from a few litres of broth containing <sup>60</sup>Co by the following steps: adsorption on charcoal; washing with 6 % phenol and elution with 60 % acetone; fractionation with butanol and ammonium sulphate; extraction with a one-to-two mixture of phenol and benzene from alkaline solution; precipitation with ether from this extract and again from an alcoholic solution of the precipitate; crystallization from aqueous acetone.

#### Chemistry of the B<sub>12</sub> vitamins

All that is so far known about the constitution of vitamin B<sub>12</sub> can be represented by the following partial formula:



This shows 5:6-dimethylbenzimidazole in glycoside linkage with phosphorylated ribose. All this part of the molecule can be split off by mild acid hydrolysis, leaving a red acidic substance of unknown constitution containing firmly co-ordinated trivalent cobalt. Other hydrolytic fragments are ammonia and two molecules of optically active 1-amino-2-propanol.

Vitamin B<sub>12</sub> itself has a cyano-group linked to the cobalt atom; the valencies are internally satisfied and the molecule as a whole is neutral, apart from several very weakly basic groups. All the other B<sub>12</sub> vitamins arise by replacement of CN by other groups and they can all be reconverted into vitamin B<sub>12</sub> by treatment with cyanide. Thus vitamin B<sub>12a</sub> (or vitamin B<sub>12b</sub>) carries a hydroxyl group, or more usually a neutral water molecule, so that the whole molecule becomes basic. It is formed when the cyano group is removed from vitamin B<sub>12</sub> by exposure to visible light or by treatment with hydrogen and a catalyst or with certain other reducing agents. Vitamin B<sub>12c</sub> carries a nitrite group, and other acid radicals that co-ordinate readily can be introduced, such as thiocyanate and cyanate. Unstable acidic adducts are known containing two such acid ions (Smith, 1952). The name cyanocobalamin has been proposed for vitamin B<sub>12</sub> and corresponding names for related compounds.

#### *Physical properties of vitamin B<sub>12</sub>*

Vitamin B<sub>12</sub> occurs in deep-red crystals containing a variable amount of water of crystallization. It is moderately soluble in water (1.2 %) and in alcohols, but not in most other organic solvents. It is stable in the dry state, even at 100°. Neutral or faintly acidic aqueous solutions can be stored for long periods without appreciable loss, provided bacterial attack is prevented; they also withstand brief autoclaving. Slow destruction occurs at room temperature in solutions more acid than pH 2 or more alkaline than pH 9. Vitamin B<sub>12</sub> is optically active. It has a characteristic absorption spectrum showing main maximums at 278 and 361 m $\mu$  in the ultraviolet and at 525 and 550 m $\mu$  in the visible range. The absorption spectrums of the other B<sub>12</sub> vitamins are similar, but the maximums are slightly displaced.

#### *Bound vitamin B<sub>12</sub>*

In natural sources vitamin B<sub>12</sub> may occur loosely bound to protein or perhaps to other substances. Some, if not all, of these bound forms are unavailable to micro-organisms used for assay purposes. The vitamin B<sub>12</sub> is easily freed by heat treatment or with proteolytic enzymes.

Combination between vitamin B<sub>12</sub> and some component of gastric juice can be demonstrated in vitro. Microbiological activity disappears wholly or in part but is largely restored on boiling. The complex is non-dialysable. It has been widely assumed that this 'vitamin B<sub>12</sub>-binding component' is identical with Castle's intrinsic factor, the substance in gastric juice that promotes the absorption of orally administered vitamin B<sub>12</sub>. This conclusion appears, however, to be premature, because the two factors exhibit some differences in behaviour and, moreover, binding of vitamin B<sub>12</sub> can be demonstrated with solutions of some proteins that have no intrinsic-factor activity.

Some new factors closely related to vitamin B<sub>12</sub> have recently been obtained in pure or nearly pure form, on both sides of the Atlantic (Pfiffner, Calkins, Peterson, Bird, McGlohon & Stipek, 1951; Coates, Ford, Harrison, Kon, Porter, Cuthbertson & Pegler, 1951; Ford, Kon & Porter, 1951; Wijmenga, 1951). Their chemistry has not yet been elucidated, but it has been suggested they may be precursors in the bacterial synthesis of vitamin B<sub>12</sub>.

#### *Chemical and physical methods of assay*

Sufficiently pure concentrates can be assayed directly with a colorimeter or a spectrophotometer. The red cobalt-containing fragment split off on acid hydrolysis can readily be esterified and separated from more water-soluble impurities. It can then be estimated colorimetrically or ashed for colorimetric cobalt determination with one of the nitrosonaphthols. The benzimidazole fragment can be removed by drastic hydrolysis and converted into either a coloured or an intensely fluorescent derivative. Finally HCN can be liberated by photolysis and estimated by a sensitive colorimetric reaction with pyrazolone.

#### *Biological assays*

Several microbiological-assay techniques have been described, using various lactobacilli or a *Bacterium coli* mutant. Hutner, Provasoli, Schatz & Haskins (1950), who devised an assay method with *Euglena gracilis*, have recently described another with either the Pringsheim chryomonad strain or *Poterochromonas stipitata*, which should respond directly to bound forms of vitamin B<sub>12</sub> (Hutner, 1951).

Assay methods on chicks, rats and mice have also been described. The vitamin B<sub>12</sub> requirement of these animals can be exaggerated by the use of diets high in vegetable protein or containing thyroxin or thyroid powder. It is usually necessary, or preferable, to minimize the vitamin B<sub>12</sub> stored by the test animals, by rearing them from females on restricted diets.

#### *Animal and human requirements*

It is abundantly clear that vitamin B<sub>12</sub> is much more than a specific treatment for macrocytic anaemias. It is undoubtedly required in minute amounts by many, if not all, higher animals. The need is difficult to demonstrate in some species, particularly in ruminants, owing to vigorous bacterial synthesis in the rumen or alimentary tract. The human requirement is normally met from dietary sources, although beneficial results have been claimed from administration of vitamin B<sub>12</sub> in a number of conditions, and in particular for physically backward children. Pernicious anaemia appears to arise not from shortage in the diet, but from impairment of the ability to absorb vitamin B<sub>12</sub>.

#### *Origin and distribution*

Vitamin B<sub>12</sub> may well be unique in being synthesized exclusively by microorganisms. Its presence in traces in soil, pond water and the roots of some plants could be explained in this way. It has not been demonstrated with certainty in vegetable tissues other than hair roots. The tissues of herbivorous animals probably

derive their vitamin B<sub>12</sub> by absorption of microbially synthesized material from the gut. Carnivorous animals may derive theirs partly in this way and partly from their food.

Vitamin B<sub>12</sub> has been found in significant amounts in nature only in fermented materials like faeces and in animal products rich in protein. Liver and kidney appear to be the only relatively rich sources and contain around 0.5 p.p.m. Other meats, egg yolk, cheese and casein contain only a few parts per 100 million.

For literature references, see reviews on vitamin B<sub>12</sub> (Smith, 1950-1; Ungley, 1951-2).

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### The Pathogenesis of Megaloblastic Anaemias and the Value of Vitamin B<sub>12</sub>

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#### *Origins of megaloblastic anaemias*

After the discoveries of Minot & Murphy (1926) and Castle (1929) earlier toxic theories were discarded and all megaloblastic anaemias were attributed to either simple or 'conditioned' deficiencies of an active principle present in food and stored in the liver. The subsequent isolation of vitamin B<sub>12</sub>, folic acid and the citrovorum factor further strengthened current beliefs in the purely nutritional origin of this group of anaemias, although dissenting voices had been raised by Dock (1938) and Bomford (1946). In this paper nutritional and toxic theories are welded into a single working hypothesis.

The main dietary sources of vitamin B<sub>12</sub> are of animal origin: organ meats, muscle meats, fish, milk and eggs. The folic-acid group of substances (including citrovorum factor) comes not only from animal sources but from fruits and green leaves. Yeast is a source of the folic-acid group and of the hypothetical Wills's factor, if this is a separate entity. Moreover, although lacking vitamin B<sub>12</sub>, some yeast extracts have the properties of an extrinsic factor of Castle in that their haemopoietic effects in pernicious anaemia are potentiated by the simultaneous oral administration of a source of intrinsic factor (Strauss & Castle, 1932; Ungley & Moffett, 1936). A deficient intake