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Present Knowledge of the Metabolic Role of Vitamin B_{12} and Related Compounds, with Particular Reference to the Role of Cobalt in Ruminant Metabolism

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The chemistry of vitamin B_{12}, its determination by microbiological assay, its relation to anaemia and its nutritional significance as part of the animal protein factor were discussed at a Nutrition Society symposium last January (Smith, 1952; Ford, 1952; Ungley, 1952; Girdwood, 1952; Cuthbertson, 1952; Coates, 1952). This paper presents some of the evidence now available concerning the ways in which vitamin B_{12} functions in metabolic processes in higher animals and in micro-organisms. The topics discussed have been restricted to those in which vitamin B_{12} has a well authenticated role. The later sections include a discussion of the occurrence of cobalt-containing compounds related to vitamin B_{12} but not members of the cobalamin series, and their possible function in the growth and metabolism of the microbial flora of the gut.

Deficiency of vitamin B_{12} in man, caused either by impaired absorption or by a dietary deficiency, results in megaloblastic anaemia. In other animals deficiency of this vitamin retards growth, but does not, necessarily, cause an anaemia. Most bacteria are able to cover by synthesis their requirements of vitamin B_{12}, but under certain conditions some lactobacilli, mutants of *Bacterium coli* and the protozoa, *Euglena gracilis* and some chrysomonads, require the vitamin for growth.

Studies with rats and chicks, and with micro-organisms, have suggested that vitamin B_{12} may be required as a coenzyme for a number of synthetic processes.

The metabolic role of vitamin B_{12} in animals

Vitamin B_{12} and one-carbon fragments

Vitamin B_{12} and pteroylglutamic acid are closely associated in haematopoiesis (Ungley, 1952; Girdwood, 1952). They are similarly associated in many metabolic processes, particularly so in the intermediary metabolism of one-carbon fragments, for there is much circumstantial evidence that pteroylglutamic acid, in its ' biologically active ' form, folinic acid, acts as a carrier of formyl groups. Vitamin B_{12} appears to be concerned with both the synthesis and the transfer of methyl groups.

Formation of methyl groups. Evidence is available, much of it from studies with ^{14}C, that the rat is able to synthesize the labile methyl groups of methionine and choline from formate and formaldehyde (Sakami & Welch, 1950; du Vigneaud, Verly, Wilson, Rachele, Ressler & Kinney, 1951; Siekevitz & Greenberg, 1950), from methanol (du Vigneaud & Verly, 1950; Arnstein, 1951), from glycine and serine (Weissbach, Elwyn & Sprinson, 1950; Jonsson & Mosher, 1950 ; Arnstein, 1951), and from acetone (Sakami, 1950). Though all these substrates allow the formation of methyl groups when pteroylglutamic acid and vitamin B_{12} are available, little is known of the importance of any particular intermediate as a source of methyl groups during normal metabolism, and such conversions may be so slow in certain species as to be nutritionally ineffective (cf. Jukes & Stokstad, 1951*a*). Studies with rats show that the utilization of the α-carbon atom of glycine for the synthesis of both moieties of choline is reduced in vitamin B_{12} deficiency, but that the utilization of the β-carbon of serine is unaffected (Arnstein & Neuberger, 1951, 1952; Stekol, Weiss & Weiss, 1952). Arnstein & Neuberger (1951, 1952) found that less than one methyl group/molecule of choline formed was derived from glycine, and concluded that the α-carbon of glycine is not converted directly to a one-carbon precursor of methyl groups. They suggested that, since vitamin B_{12} is not concerned with the formation of choline from serine, it may be involved in the conversion of glycine to a form suitable for the acceptance of formate in a serine synthesis.

Such a mechanism can also explain the toxicity of glycine to chicks receiving a vitamin B_{12}-deficient diet (Menge & Combs, 1950; Hsu & Combs, 1952; Machlin, Lankenau, Denton & Bird, 1952), as this toxicity is overcome by vitamin B_{12}.

Transmethylation. The existence of a transmethylating process within animal tissues was first postulated by du Vigneaud, Chandler, Moyer & Keppel (1939)

to explain the findings that under certain conditions homocysteine could not replace methionine in the diets of growing rats unless choline was supplied to provide the methyl groups.

Several groups of workers have demonstrated that in rats and chicks vitamin B_{12} spares methyl groups, or, alternatively, that the requirement for vitamin B_{12} is reduced by ample supplies of choline (cf. Coates, 1952; Jukes & Stokstad, 1951*a*). However, neither choline nor methionine can completely replace vitamin B_{12} in the diet of chicks depleted of it (Jukes & Stokstad, 1951*b*).

An interesting, indirect method of assessing the amount of methionine formed by transmethylation has been used by Liener & Schultze (1952). Working from the hypothesis that the methyl groups, required for the methylation of nicotinamide to N-methylnicotinamide and of guanidoacetic acid to creatine, are derived directly from methionine (Borsook & Dubnoff, 1947), they used the excretion of N-methylnicotinamide and creatinine as measures of the synthesis of methionine by rats receiving diets free from labile methyl groups and supplemented with combinations of homocystine, choline, betaine, formate and vitamin B_{12}. They found that only when the basal ration was supplemented with homocystine in the presence of choline, betaine or vitamin B_{12}, were growth and excretion of N-methylnicotinamide similar to those observed when the diet was supplemented with methionine. Formate was not an effective methyl donor to homocystine in the absence of vitamin B_{12}, though when the vitamin was present methylation did occur, and a substantial excretion of N-methylnicotinamide took place. The methylation of guanidoacetic acid proved to be an unsatisfactory measure of methionine formation, since both choline and betaine could act as direct methyl donors.

These experiments demonstrate a role for vitamin B_{12} both in transmethylation from choline or betaine to homocystine and in the synthesis of the methyl group from formate (cf. p. 107).

The precise role of vitamin B_{12} in transmethylation has not been elucidated. It may facilitate the utilization of methyl groups by catalysing the reduction of homocystine to homocysteine (Dubnoff, 1950). This concept is supported by the observation that the blood of vitamin B_{12}-deficient rats contains less thiol groups than that of litter-mates receiving vitamin B_{12} (Ling & Chow, 1951).

Vitamin B_{12} and protein metabolism

The role of vitamin B_{12} in protein metabolism is at present ill defined, but a relationship probably exists.

Several groups of workers have shown that a deficiency of vitamin B_{12} causes the accumulation of non-protein nitrogen in the blood (McGinnis, Hsu & Graham, 1948; Zucker & Zucker, 1948). Such experiments suggest that the vitamin may aid protein synthesis in the tissues.

Henry & Kon (1951) found that the biological value of casein, and hence the assimilation of nitrogen, were significantly lower in rats deprived of vitamin B_{12} than in rats receiving it. This does not necessarily prove a specific relationship

with protein metabolism in general, for it is possible that only the connexion between the vitamin and methionine formation was involved.

A protein-sparing action for vitamin B_{12} has been proposed from studies of the effect of thyroxine in vitamin B_{12} deficiency. The addition of thyroid or iodinated casein to diets containing a high proportion of vegetable protein has been used for several years to hasten the development of vitamin B_{12} deficiency in rats. Vitamin B_{12} probably acts by raising food consumption since it does not lower the basal metabolic rate (Meites & Shay, 1951). Rupp, Paschkis & Cantarow (1951) found that when the food intake was kept constant by forced feeding, vitamin B_{12} did not prevent loss of body-weight, but the loss of nitrogen caused by the katabolic action of thyroxine was reduced, indicating a sparing of protein at the expense of other body constituents.

In considering the possible connexion between vitamin B_{12} and such metabolic processes as the utilization of protein it is well to remember that the absence of any essential food factor, by interfering with the normal metabolic chain, may lead to a less efficient utilization of nutrients (cf. Kon, 1931).

Role of vitamin B_{12} in nucleic-acid synthesis in animals

Vitamin B_{12} is associated with the synthesis of deoxyribosides in lactobacilli (p. 110). There is some evidence that the vitamin may be concerned in the metabolism of thymine and in the synthesis of its derivatives in man and the pig, since haematopoietic responses have been reported with large oral doses of thymine (cf. Girdwood, 1952), and Hausmann (1951) found that two patients suffering from pernicious anaemia responded to thymidine.

Besides suggesting that vitamin B_{12} may be involved in deoxyriboside synthesis in animals (see also Rose & Schweigert, 1952), these findings indicate that deoxyribosides may be important for haematopoiesis.

Vitamin B_{12} in bacterial metabolism

Coliform organisms

General. Davis & Mingioli (1950) have isolated a number of mutants of *Bact. coli* that required vitamin B_{12}. All such mutants also responded to methionine, and, conversely, mutants requiring methionine were found to respond to vitamin B_{12}. Homocysteine could not replace methionine, even when methylating agents such as choline and betaine were present, and the authors suggested that in these mutants the synthesis of methionine was blocked at the methylation of homocysteine.

Dubnoff (1952) reinvestigated the replacement value of homocysteine for a *Bact. coli* mutant and showed that under anaerobic conditions this compound can support growth, but optimal growth was not obtained unless a trace of vitamin B_{12} was also present. Under aerobic conditions homocysteine added to the medium was oxidized to homocystine, which did not support growth of the mutant. Dubnoff

suggests that vitamin B_{12} may have a dual role in methionine synthesis in *Bact. coli* mutants. Firstly, in maintaining homocysteine in the reduced condition and, secondly, in the synthesis of the methyl group required for its methylation.

Vitamin B_{12} and p-*aminobenzoic acid.* A relationship between these vitamins has only been demonstrated with coliform organisms. Studies with wild types of *Bact. coli* inhibited by sulphonamide (Shive, 1950; Davis & Mingioli, 1950) and with mutants of *Bact. coli* requiring *p*-aminobenzoic acid (Lampen, Jones & Roepke, 1949; Lampen, 1950; Davis, 1951) have given broadly similar results. Vitamin B_{12} or methionine reduced the requirements of *Bact. coli* mutants for *p*-aminobenzoic acid. In harmony with this finding, sulphonamides inhibited primarily methionine formation, and the minimum inhibitory concentration was greatly increased in the presence of vitamin B_{12} or methionine. This relationship was non-competitive. Davis (1951) interpreted these results as suggesting that either *p*-aminobenzoic acid is used in the synthesis of vitamin B_{12}, a view that he has since modified (Davis, 1952), or that *p*-aminobenzoic acid has a catalytic function in vitamin B_{12} synthesis.

Possibly a more attractive hypothesis, and one that fits the facts equally well, is that vitamin B_{12} catalyses the conversion of *p*-aminobenzoic acid to a coenzyme (Eakin, 1950).

Lactobacilli

Under specified conditions a number of lactobacilli (*Lactobacillus bifidus*, *Lb. lactis*, *Lb. leichmannii*) require vitamin B_{12} for growth. Hoffmann, Stokstad, Franklin & Jukes (1948) showed that thymidine could replace vitamin B_{12} for *Lb. leichmannii*, and it is now recognized that several deoxyribosides can replace vitamin B_{12} in the nutrition of these organisms. However, thymine cannot replace thymidine, and thymidine itself is not a substitute for vitamin B_{12} unless other purine bases are present in the medium (cf. Downing, Rose & Schweigert, 1952).

Vitamin B_{12} is closely connected with pteroylglutamic acid in the synthesis of nucleosides by microbes and by higher animals, according to the following scheme:

$$\text{precursors} \xrightarrow{\text{folinic acid}} \begin{matrix}\text{purines}\\ \text{pyrimidines}\end{matrix} \xrightarrow{\text{vitamin } B_{12}} \text{nucleosides.}$$

The fact that other deoxyribosides can replace thymidine is believed to indicate that in the presence of other purines growth occurs as a result of the transfer of deoxyribosides from one pyrimidine or purine to another (MacNutt, 1950). No satisfactory mechanism for such changes has been elaborated.

Euglena gracilis *and Chrysomonads*

When growing on simple media these organisms need neither methionine nor deoxyribosides. The function of vitamin B_{12} in the growth of these organisms is not yet known.

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Vitamin B_{12}-like compounds

During the past 2 years several compounds possessing vitamin B_{12} activity for micro-organisms have been isolated. These compounds are related to, but are not members of, the cobalamin series. Wijmenga (1951) isolated from pig faeces a crystalline substance he named vitamin B_{12m}. Ford & Porter (1952) showed that the faeces of ruminating calves contained vitamin B_{12}-like factors, A, B and C, in addition to vitamin B_{12} itself; Pfiffner, Calkins, Peterson, Bird, McGlohon & Stipek (1951) obtained pseudovitamins B_{12} and B_{12b} from a rumen anaerobe, and Lewis, Tappan & Elvehjem (1952) reported that rat faeces contained a substance they called vitamin B_{12f}.

Through the generosity of Dr. Wijmenga and Dr. Pfiffner we have been able to examine their compounds and compare them with those isolated at Shinfield. The results of these findings have been published (Holdsworth, 1953; Ford, 1953; Ford, Holdsworth, Kon & Porter, 1953). Briefly, we have shown by ionophoresis at pH 2·5, and differential microbiological tests, that none of the compounds isolated and considered as being pure were in fact pure, but that each of them contained one or more of the others as impurities. Thus vitamin B_{12m} and factor A contain the same major component (factor A), but also vitamin B_{12} and pseudovitamin B_{12}. Pseudovitamins B_{12} and B_{12b} contain the same major component (pseudovitamin B_{12}) but also some factor A, and pseudovitamin B_{12b} contains in addition a small amount of material that is almost inactive microbiologically. It is clear from the microbiological and ionophoretic findings that factors A (vitamin B_{12m}) and pseudovitamin B_{12} are different substances.

We now consider in the light of these and other findings (Ford *et al.* 1953) that the vitamin B_{12} activity of extracts of gut contents and faeces, prepared in the presence of cyanide, is contributed in varying proportions by the following five substances: factors A (vitamin B_{12m}), B and C, pseudovitamin B_{12}, and vitamin B_{12} itself (cyanocobalamin).

These compounds do not appear in body tissues so far examined to any appreciable extent, though small amounts of each have been isolated from Wijmenga's factor WR, prepared from beef liver.

Metabolic role of vitamin B_{12}-active compounds other than vitamin B_{12}

Bacteria

Bact. coli. Factors A, B, and C and pseudovitamin B_{12} all promote growth of *Bact. coli* (cf. Ford, 1953), the general order of activity being similar to that of vitamin B_{12}.

As they are active for *Bact. coli*, it is reasonable to suppose that the compounds are capable of replacing vitamin B_{12} in the synthesis of methionine, and though we know that they differ in certain respects from vitamin B_{12} in molecular structure (e.g. pseudovitamin B_{12} contains adenylic acid in place of the benziminazole

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nucleotide (Dion, Calkins & Pfiffner, 1952) they are similar in that they all contain the cyano-group and can form cyanide adducts. It is tempting to use this evidence to support the suggestion by Dubnoff (1951) that a cyanolysis step is involved in methionine synthesis.

Lactobacilli. Factor A and pseudovitamin B_{12} are differently active for *Lb. leichmannii*, though both rather less so than vitamin B_{12}. Factor B is inactive and factor C only slightly active (Ford, 1953; Ford & Porter, 1952).

It is apparent, therefore, that factor A and pseudovitamin B_{12} can replace vitamin B_{12} in those reactions concerned with deoxyriboside formation, whereas factor C can do so to a limited extent, and factor B not at all.

Animals

Preliminary tests on factor A and vitamin B_{12m} suggested that those compounds had some biological activity for chicks and for man (Coates, Harrison, Ford, Kon & Porter, 1952; Wijmenga, 1951). As we now know that the materials used were slightly impure, particularly in that they contained some vitamin B_{12}, we are doubtful of the validity of these earlier tests, and they are being repeated. It is perhaps significant that the chick test showed an activity for factor A about one-twentieth of that of vitamin B_{12}, and that factor A, on ionophoretic separation, yielded 5% of vitamin B_{12}.

Cobalt in ruminant nutrition

Deficiency of cobalt leads to a wasting disease in ruminants, recognized and described in various areas in many parts of the world (Marston, 1935, Underwood & Filmer, 1935). Marston (1952) has recently published an extensive review on the role of cobalt in nutrition, and it is unnecessary here to recapitulate the earlier work, the significant finding of which was that cobalt was effective only by mouth, suggesting that its site of function was the rumen. It will suffice to say that when vitamin B_{12} was isolated and found to contain cobalt it occurred to a number of workers to examine its effect on cobalt-deficient ruminants.

Earlier experiments with cobalt-deficient sheep given vitamin B_{12} by mouth or parenterally yielded negative results. The amounts given were of the same order as those used in the treatment of pernicious anaemia in man.

Later, doses twenty times greater (300 μg vitamin B_{12}/sheep/week) were immediately effective (cf. Marston & Lee, 1952).

It is quite clear from these studies that vitamin B_{12} cures the signs of cobalt deficiency in ruminants, but it is not yet proved that it is the only cobalt-containing compound that is of importance in the ruminant.

During normal feeding, when the diet contains a reasonable supply of cobalt, rumen contents and faeces contain up to 10μg vitamin B_{12} activity/g dry matter, as measured by *Bact. coli* assay; of this quantity only about 1 μg/g is vitamin B_{12}. Factor A supplies about 6 μg/g and pseudovitamin B_{12} and factors B and C the remainder (cf. Ford *et al.* 1952). In cobalt deficiency the vitamin B_{12} activity

of rumen contents naturally falls very greatly (to about 0·5-1·0 μg/g, as measured by *Bact. coli* assay, but is still the major contributing component (Porter, unpublished results)*. These findings conflict with those of Dawbarn, Hine & Hughes (1952), who by differential assays with *Bact. coli* and *Lb. leichmannii* found in faeces from cobalt-deficient sheep a decreased ratio of *coli : leichmannii* activity, a result that would suggest that more vitamin B_{12} and less of other factors was present.

Be it as it may, the normal rumen contains large amounts of factors other than vitamin B_{12}, and it is reasonable to suppose, as we have already suggested (Ford *et al.* 1952), that these other factors may be necessary for normal microbial function in the rumen, whereas vitamin B_{12} is clearly essential for the normal metabolism of the animal itself. The relatively massive doses of vitamin B_{12} required to cure cobalt deficiency in ruminants may be needed either because the tissues of the animal have an exceptionally great demand for the vitamin, or because some of the dose must find its way to the rumen before the condition is cured. Vitamin B_{12} could probably reach the rumen from the blood stream either through the saliva or by passage through the rumen wall.

Once in the rumen, vitamin B_{12} can be used by the micro-organisms either as vitamin B_{12} or by conversion to one of the other vitamin B_{12}-active compounds. Gall & Huhtanen (1951) have shown that the rumen flora is changed in cobalt deficiency, and it is clearly possible that a lack of cobalt prevents the synthesis of cobalt-containing compounds necessary to sustain certain organisms.

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Recent Studies on Vitamin K

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The term vitamin K designates a group of methylnaphthoquinone derivatives that prevent a haemorrhagic state due to defective clotting of the blood. Vitamin K_1 from green leaves, phylloquinone, is 2-methyl-3-phytyl-1:4-naphthoquinone. Vitamin K_2 from bacteria has a difarnesyl residue instead of the phytyl side chain.

The artificially produced 2-methyl-1:4-naphthoquinone (menaphthone, menadione) and certain of the water-soluble esters of its hydroquinone are commonly used instead of the more expensive naturally occurring vitamin K_1 and K_2. The list of related compounds with more or less pronounced vitamin K activity is comprehensive.