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The Microbiological Assay of Vitamin B₁₂

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The literature on the microbiological assay of vitamin B₁₂ is large and growing. Those familiar with microbiological techniques realize their limitations, but there is a danger that the apparent ease with which the tests can be carried out may encourage uncritical acceptance of the spate of results now appearing. An object of this paper is to show some of the difficulties that may be involved in the assay of vitamin B₁₂.

To begin with there are the ever-present fundamental problems of specificity of test and completeness of extraction. There is next the problem of the total extraction and measurement as vitamin B₁₂ of the series of related substances, all active micro-biologically, that may be present in natural products, and that are distinguished from vitamin B₁₂ only by the replacement of the cyano-group by a variety of complex-bound anions (Brink, Kuehl & Folkers, 1950; Kaczka, Wolf, Kuehl & Folkers, 1950; Smith, 1948; Smith, Fantes, Ball, Ireland, Waller, Emery, Anslow & Walker, 1951; Pierce, Page, Stokstad & Jukes, 1949; Wijmenga, Veer & Lens, 1950).

A further problem arises from the presence in some natural materials of other

substances also related to vitamin B₁₂ but differently active for different assay organisms. They are not in the strict sense cobalamins and cannot be converted to vitamin B₁₂ by treatment with cyanide (Coates, Ford, Harrison, Kon, Porter, Cuthbertson & Pegler, 1951; Coates, Ford, Harrison, Kon & Porter, 1952; Ford, Kon & Porter, 1951; Ford & Porter, 1952; Pffiffer, Calkins, Peterson, Bird, McGlohon & Stipek, 1951; Wijmenga, 1951).

Finally, vitamin B₁₂ may also be present in some natural materials in a micro-biologically inactive, 'bound' form (Bird & Hoevet, 1951; Smith, 1950-1; Meyer, Eppstein, Bethell & Hall, 1950; Shaw, 1950; Ternberg & Eakin, 1949).

It will be helpful to begin with a brief summary of some of the more commonly used microbiological methods.

The lactobacilli as test organisms

Under specified conditions, several of the lactic-acid bacteria require vitamin B₁₂. Of these, strains of *Lactobacillus leichmannii* and *Lb. lactis* are widely used to assay the vitamin. The requirement for vitamin B₁₂ is not specific, in the sense that in the nutrition of these organisms certain nucleic-acid derivatives or deoxyribonucleic acid can replace, or stimulate the response to, vitamin B₁₂. The effects of these substances can, however, be minimized and often altogether eliminated, particularly in tube assays, by the use of appropriate techniques (see review by Snell & Wright, 1950 and Skeggs, Nepple, Valentik, Huff & Wright, 1950).

Vitamin B_{12b}, one of the cobalamins present in natural materials, is readily inactivated for *Lb. leichmannii* on heating with the assay medium (cf. Soars & Hendlin, 1951) and may thus be lost in the process of measurement. Vitamin B_{12b} is readily converted into vitamin B₁₂ by treatment with cyanide (Brink *et al.* 1950; Ellis, Petrow, Beaven, Holiday & Johnson, 1950; Veer, Edelhausen, Wijmenga & Lens, 1950) and the presence of minute amounts of cyanide prevents inactivation of vitamin B_{12b} on heating with the assay medium, apparently by converting it to the stable vitamin B₁₂ (Cooperman, Drucker & Tabenkin, 1951; Soars & Hendlin, 1951). Cooperman *et al.* found with some products that only with media containing cyanide did *Lb. lactis* assays agree with those by *Lb. leichmannii*.

Assays with Bacterium coli

A mutant of *Bact. coli* requiring vitamin B₁₂ is now increasingly used in a plate method perfected by workers at the Glaxo Laboratories (Bessell, Harrison & Lees, 1950; Cuthbertson, Pegler & Lloyd, 1951; Harrison, Lees & Wood, 1951). The method is speedy and simple in operation and allows large numbers of assays to be carried out on a routine basis. It is subject to interference by methionine and to a lesser extent by other substances, but in general such interference is readily detected from the appearance of the zones of exhibition.

Burkholder (1951) has described the use of this organism in a tube assay and reported good agreement between assays of a number of substances with it and with *Euglena gracilis*. This tube-assay technique is more sensitive than the plate method, which is best suited to the assay of higher-potency materials and extracts.

Assays with Euglena gracilis

The high sensitivity and apparent specificity of *Euglena gracilis* as compared with other assay organisms (Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin & Jukes, 1949; Picken & Bauriedel, 1950) has recommended its use with low-potency materials. Robbins, Hervey & Stebbins (1950, 1951) have used it to measure the vitamin B₁₂ content of a wide range of natural materials, Ross (1950) for body fluids and liver extracts, but Picken & Bauriedel (1950) found the method satisfactory only for the assay of relatively pure preparations. They described a method of using the organism, together with *Lb. leichmannii*, in comparative bioautographic studies of vitamin B₁₂ and related growth factors.

Experience at Shinfield

It has been generally accepted that these three types of organism can be used for the measurement of vitamin B₁₂ itself, and for some time now we have been using all three for work in our laboratory. We soon observed, however, that they respond differently to certain vitamin B₁₂-like factors, and we now use them in conjunction as a means of differentiation between these factors.

For assays with *Lb. leichmannii* our method and medium are essentially as described by Skeggs *et al.* (1950) but with certain minor modifications in technique. In our experience this method measures in a reproducible fashion those alkali-labile substances in cyanide extracts of a wide variety of natural materials active for *Lb. leichmannii*.

Our *Bact. coli* method is that of the Glaxo workers (Bessell *et al.* 1950; Cuthbertson *et al.* 1951; Harrison *et al.* 1951). With exceptions discussed below, we find broad similarity between results obtained with *Bact. coli* and *Lb. leichmannii*, and for certain crude and refined liver extracts agreement is good.

For the *Euglena gracilis* assays we use a technique based on that of Hutner *et al.* (1949). Results obtained for liver extracts agree broadly with those obtained with *Bact. coli* and *Lb. leichmannii*, but in general reproducibility is not satisfactory. In our hands the method has so far proved incapable of precisely measuring the vitamin B₁₂ activities of liver preparations, fish and whale products and gut contents and faeces. We have used the method allied with chromatography to indicate qualitatively the presence of vitamin B₁₂ activity in extracts.

Properties of some microbiologically active substances related to vitamin B₁₂

Natural materials may contain substances of the vitamin B₁₂ group active for one or more assay organisms (Coates, Ford, Harrison, Kon & Porter, 1952; Ford *et al.* 1951; Ford & Porter, 1952; Kaczka *et al.* 1950; Smith *et al.* 1951; Piffner *et al.* 1951; Pierce *et al.* 1949; Tarr, 1951; Wijmenga, 1951). Further compounds closely related to vitamin B₁₂ have been prepared in the laboratory (Cooley, Ellis, Petrow, Beaven, Holiday & Johnson, 1951; Kaczka *et al.* 1950; Smith *et al.* 1951). Though most of these substances can be converted to vitamin B₁₂ by the action of cyanide, we have found in calf gut-contents and faeces, in addition to vitamin B₁₂, three substances, provisionally described as fractions A, B and C, related to vitamin B₁₂ but having widely different activities for different assay organisms, and not convertible

by cyanide (Coates, Ford, Harrison, Kon & Porter, 1952; Ford *et al.* 1951; Ford & Porter, 1952). Fractions A and B are red, cobalt-containing pigments. Fraction C is found in calf faeces but has not yet been isolated from this source. We have prepared it by growing *Bact. coli* in a vitamin B₁₂-free medium supplemented with fraction B. The vitamin B₁₂ activity of extracts of the harvested cells was in the form of fraction C. Both fraction A and vitamin B₁₂ were recovered largely unchanged after such passage through *Bact. coli*.

Fraction A is about as active for *Bact. coli* and *Euglena gracilis* as vitamin B₁₂. It is the most abundant active component in cyanide extracts of calf faeces and is approximately five times more active for *Bact. coli* and *Euglena gracilis* than for *Lb. leichmannii*. Modification of the *Lb. leichmannii* assay-medium by omission of thiomalic acid and inclusion of sodium cyanide (cf. Soars & Hendlin, 1951) results in an apparent marked increase in the activity of fraction A relative to vitamin B₁₂.

Fraction B is probably as active for *Bact. coli* as vitamin B₁₂, but it has less than $\frac{1}{2000}$ of this activity for both *Lb. leichmannii* and *Euglena gracilis*, and may be quite inactive for them. There is marked non-parallelism in the *Bact. coli* assay of this substance in terms of vitamin B₁₂.

As far as we can judge, fraction C is as active for *Bact. coli* as fraction B and in crude extracts has about one-fifteenth this activity for *Lb. leichmannii*.

Fractions B and C produce similar and characteristic zones of exhibition on *Bact. coli* plates. They are less sharply defined than those characteristic of fraction A and vitamin B₁₂.

We have prepared a derivative of fraction A apparently analogous to vitamin B_{12b} (Ford & Porter, 1952). It may well be that further compounds exist, related to fractions A, B or C in the same way as the cobalamin series is related to vitamin B₁₂.

Cyanide treatment of these three fractions had no effect on their chromatographic behaviour or on their various microbiological potencies.

Fractions A and B are inactive for the chick when given in the diet, but fraction A is active by injection, and on clinical test was shown to be about as active as vitamin B₁₂ in a single case of pernicious anaemia (Coates, Ford, Harrison, Kon & Porter, 1952).

Pfiffner *et al.* (1951) have isolated a rumen organism that in anaerobic fermentation produced a mixture of cobalt-containing pigments of the vitamin B₁₂ group. They crystallized two of these substances, which were active for *Lb. leichmannii* and *Lb. lactis* but inactive in chick and rat tests.

Wijmenga (1951) obtained a compound, vitamin B_{12m}, from the faeces of pigs, horses and cattle. Its absorption spectrum was similar to that of vitamin B₁₂, but it was slower-moving on paper chromatograms, and on microbiological assay with *Lb. leichmannii* had only about half the activity of vitamin B₁₂. Vitamin B_{12m} is a cyano-complex, and strong illumination of a solution in 0.001N-H₂SO₄ gave rise to a substance of smaller R_F value. This derivative was designated vitamin B_{12mb}.

Another substance, 'factor WR', was isolated from liver. Its absorption spectrum coincided with that of vitamin B₁₂, but it behaved very differently in paper partition-chromatography, and had only 40% of the microbiological activity (*Lb. leichmannii*) of vitamin B₁₂.

Effect of cyanide in facilitating extraction of vitamin B₁₂-active substances from natural materials

De Heus & De Man (1951) have measured microbiologically the vitamin B₁₂ potencies of a number of substances extracted in the presence and absence of cyanide. In several instances they obtained much higher results with cyanide. In our experience cyanide facilitates extraction of the vitamin B₁₂-active components of gut contents and faeces, and gives higher and more reproducible results than those obtained without cyanide. Cyanide treatment did not increase the potencies of extracts, whereas its addition before extraction gave extracts of greatly increased potencies (Coates, Ford *et al.* 1951).

Wijmenga *et al.* (1950) showed that cyanide can replace proteolytic enzymes in the extraction of vitamin B₁₂ from liver, as it apparently removes a protein or peptide group, originally linked to the vitamin B₁₂ molecule. We have compared different methods of extracting vitamin B₁₂ from pig livers and found that steaming or autoclaving the homogenized livers with buffer at pH 5.5 in the presence of cyanide was as effective, as judged by *Lb. leichmannii* assay, as was autolysis followed by digestion at 65° with cyanide-activated papain. *Bact. coli* assays of the papain digests were unsatisfactory because of the large amounts of interfering substances present. No such interference was found with extracts prepared by the other methods, and with these extracts the two methods of assay gave similar results.

Assay of preparations containing mixtures of active substances that are not readily converted to vitamin B₁₂ on cyanide treatment

To assay preparations containing mixtures of active substances, it may be necessary to fractionate and assay the substances separately against appropriate standards, and chromatographic methods have obvious applications to this end. We have described a technique for the rapid separation on paper chromatograms of the vitamin B₁₂-active substances in gut contents and faeces (Ford *et al.* 1951). We have used it to study the increase in the vitamin B₁₂ potency of calf faeces following incubation, shown by chick tests. The microbiological potency for *Lb. leichmannii* changed little on incubation, whereas potency for *Bact. coli* diminished. We found that incubation resulted in a marked increase in the vitamin B₁₂ component and the disappearance of a proportion of the activity contributed by other factors, in particular fraction A. This evidence suggests a possible explanation of the discrepancies between chick and microbiological assays, and of the increased potency for chicks after incubation.

'Binding' of vitamin B₁₂

Some natural materials have the property of apparently combining with vitamin B₁₂ and making it unavailable to assay micro-organisms (Bird & Hoebet, 1951; Meyer *et al.* 1950; Shaw, 1950; Ternberg & Eakin, 1949). Sow's milk, for example, ties up considerable amounts of added vitamin B₁₂. The binding factor is not present in ultra-filtrates of sow's milk, and vitamin B₁₂ added to the milk becomes not ultra-filtrable. The apparent inhibition of growth of the assay organisms is overcome by the

addition of vitamin B₁₂ in excess of about 0.05 µg/ml. milk. The binding property is not destroyed, nor is added vitamin B₁₂ released, on autoclaving the milk for 15 min at 120°. Digestion with cyanide-activated papain, or autoclaving in the presence of a trace of cyanide, however, destroyed the 'binding' property of the milk and released added vitamin B₁₂ (Gregory, Ford & Kon, 1952).

Conclusions

The non-specificity of available techniques of microbiological assay can complicate their use with some natural materials that may contain substances related to vitamin B₁₂, but differently active for different micro-organisms. It is possible, however, that these substances are less readily utilized by higher animals than is vitamin B₁₂ itself and that the assay of animal tissues may therefore present fewer problems of non-specificity than that of fermentation products.

The use of *Bact. coli* in bioautographic techniques offers a ready means of identifying at least three substances related to vitamin B₁₂ that may be encountered in cyanide-treated extracts of natural materials. The presence of cyanide can facilitate the extraction of vitamin B₁₂-active substances from natural materials and simplify their measurement.

When microbiological assays are used to assess the value of foods for man or beast, it should be borne in mind that the response of micro-organisms to any nutrient as present in natural materials is not necessarily that of higher animals and, as with all assays, the question turns on the purpose for which the assay is carried out (Coates, Ford, Harrison, Kon, Shephard & Wilby, 1952).

Reports of marked discrepancies between results of biological and microbiological assays of vitamin B₁₂ and related substances (Coates, Harrison & Kon, 1951; Combs, Carlson, Miller, Peeler, Norris & Heuser, 1950; Lewis, Tappan & Elvehjem, 1950; Pffiffer *et al.* 1951) further emphasize the need for a biological check before interpreting the results of microbiological assays in terms of animal requirements.

With microbiological assays of vitamin B₁₂ it appears that the fullest particulars of methods of extraction and assays, with data on recovery tests and possibly also the protocols of a typical assay, may be necessary for the interpretation of published findings. To quote Harris (1949), 'It is essential that workers reporting the results of vitamin determinations should not be content with giving merely a numerical value, but should state also the conditions employed for the assay'.

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The Nutritional Significance of the Animal Protein Factor and Antibiotics

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Diets composed wholly of vegetable materials have for some time been known to be inadequate for the growth of chicks (Hammond & Titus, 1944*a, b*; Rubin & Bird, 1946) or rats (Zucker & Zucker, 1948), even when supplemented with all the previously recognized vitamins. The factor (or group of factors) essential for the growth of animals maintained on these diets came to be called the 'animal protein factor' (APF) and was soon shown, though not a protein, to be present in materials of animal origin, e.g. milk, meat, liver, fish meal and cow manure (Rubin, Groschke & Bird, 1947; Bird, Rubin & Groschke, 1948).

The growth of chicks and pigs on wholly vegetable diets was greatly improved by administration of vitamin B₁₂ (Ott, Rickes & Wood, 1948; Hagan & Anderson, 1949),