

VITAMIN B₁₂ ASSAY IN BODY FLUIDS USING *EUGLENA GRACILIS*

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BY

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The methods of assay of vitamin B₁₂ most generally used depend on the fact that some lactobacilli require this vitamin as an essential growth factor and that growth is proportional to the amount available over a certain range of concentration. Some desoxynucleotides (Shive, Sibley, and Rogers, 1951), desoxyribonucleosides and thymidine particularly, and many other substances too, have, however, been found to replace vitamin B₁₂, although in relatively large amounts only. Assays with lactobacilli then are not entirely specific and this fact, combined with rather low sensitivity, makes these methods unsatisfactory for detecting the low concentrations of vitamin B₁₂ found in some body fluids. The results of lactobacillus assays of whole blood of a number of animal species (Couch, Olcese, Witten, and Colby, 1950), of milk of a number of animal species (Collins, Harper, Schreiber, and Elvehjem, 1951), of urine of subjects receiving vitamin B₁₂ therapy (Chow, Lang, Davis, Conley, and Ellicott, 1950), and of human urine (Girdwood, 1951) have, however, been reported. Plate assays with a *Bact. coli* mutant requiring vitamin B₁₂ as a growth factor (Bessell, Harrison, and Lees, 1950) are also lacking in sensitivity, but, because of the simplicity of the method and rapidity of growth, are useful for the assay of large concentrations such as are found in urine after injection of vitamin B₁₂ (Chesterman, Cuthbertson, and Pegler, 1951). Chemical methods of assay are being developed (Boxer and Rickards, 1951; Fantes, Ireland, and Green, 1950), but are still insufficiently sensitive to detect concentrations normally found in body fluids.

The use of an alga, *Euglena gracilis* var. *bacillaris*, for the assay of vitamin B₁₂, crystalline and in liver extract, was described by Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes (1949). Assays with the *Euglena* by both the tube and plate method of a variety of material

including plants, earth, pond water, and milk have also been reported (Robbins, Hervey, and Stebbins, 1950a, b, 1951). Preliminary observations made in assays of serum, urine, and cerebrospinal fluid with the *Euglena* have been previously described (Ross, 1950).

Crystalline vitamin B₁₂, B_{12a}, and B_{12c}, and liver extracts produce a quantitative, and almost equivalent, growth response of the test organism, and the body fluids which have been tested show similar activity. No other substance has yet been shown to replace vitamin B₁₂ in the growth of the *Euglena*, and in this work it has been assumed that the *Euglena* growth-promoting effects of body fluids are, in fact, due to some form of the vitamin, and values in assays of these fluids are expressed as equivalents of vitamin B₁₂.

The present paper describes a technique of assay of serum and urine, which is also applicable to such material as liver extracts and liquid culture media.

Method

Quantitative assays have been made by comparing the growth-promoting effects of varied dilutions of the test fluids with those of known concentrations of crystalline vitamin B₁₂.

Dilutions in distilled water of test fluids and standards are added to test-tubes. Volumes are made up to 2 ml. with distilled water and 2 ml. of double-strength basal medium are added to give a final volume of 4 ml. Assay batches conveniently contain 200-250 tubes and include a complete set of standards and six control tubes with no vitamin B₁₂. The tubes are then heated in a water bath at 100° C. for 15 minutes and, after cooling, inoculation is made with one drop of a dense, actively growing stock culture of the *Euglena*, usually five to seven days old. The tubes are then incubated at 30° C. for eight to 10 days.

Heating at 100° C. for 15 minutes is essential to liberate the maximum amount of vitamin B₁₂ from the heat-labile complex in which it may be found in

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serum (Ross, 1950), and is effective in killing contaminating fungi.

An estimate of uncombined vitamin B₁₂ in these fluids can be made by setting up tubes, either unheated, or heated at only 56° C. for 30 minutes, a temperature which releases little vitamin B₁₂ from combination, yet helps to prevent contaminating growth.

Apparatus.—Test-tubes, 6 × $\frac{3}{8}$ in., plugged with cotton-wool, are used for assays, and 6 × $\frac{1}{4}$ in. tubes for maintenance of stock cultures.

The optimum temperature for the *Euglena* is 28–31° C. with inhibition effects at about 32° C. (Hutner *et al.*, 1949). The organism is rapidly killed at temperatures higher than 40° C. A perspex water tank (2 ft. 6 in. × 1 ft. 3 in. × 7 in.) heated by a test-tube immersion coil element and held at 30° C. by a Sunvic thermostat is used for incubation. An electric motor-driven water stirrer in a corner near the heating element circulates the water, and the temperature is found generally to vary from 30° C. by no more than 0.5° C. from one end of the bath to the other. Illumination is supplied from below by two 3 ft. 40-watt fluorescent strip lights which, by extending beyond the ends of the bath, maintain uniform brightness. Tubes are either bundled together at random with elastic bands or packed in rectangular partitions made of perspex. Racks are less economical of space.

A photoelectric colorimeter with a 1 cm. deep rectangular cell is used to measure turbidity of cultures.

Basal Medium.—The basal medium used is that recommended by Hutner, Provasoli, Schatz, and Haskins (1950), but with slightly different proportions of the metals. The exact amounts given in that paper and the use of Ca(NO₃)₂ in place of CaCO₃ have not been tried, but the differences in growth effect are unlikely to be significant. Hutner *et al.* (1949) found that thiamine (aneurine), although in no way replacing vitamin B₁₂, is a second essential growth factor and must be present in at least 2 μg./ml. concentration. This amount is greatly exceeded in the medium used. The composition (double strength) is as shown in the table below.

Potassium dihydrogen phosphate (KH ₂ PO ₄) 5%	..	20 ml.
l-glutamic acid	10 g.
dl-malic acid	2 "
Magnesium sulphate (MgSO ₄ ·7H ₂ O) 5%	..	20 ml.
Zinc sulphate (ZnSO ₄ ·7H ₂ O) 8·8%	..	10 "
Calcium carbonate (CaCO ₃) 1%	..	1 "
Ferrous sulphate (FeSO ₄ ·7H ₂ O) 0·3%	..	10 "
Manganese sulphate (MnSO ₄ ·H ₂ O) 1·23%	..	1 "
or (MnSO ₄ ·4H ₂ O) 1·62%	..	1 "
Cupric sulphate (CuSO ₄ ·5H ₂ O) 3·92%	..	0·1 "
Cobalt sulphate (CoSO ₄ ·7H ₂ O) 1·73%	..	0·1 "
Sodium molybdate (Na ₂ MoO ₄ ·2H ₂ O) 1·01%	..	0·1 "
Boric acid (H ₃ BO ₃) 1·41%	..	2 "
Aneurine hydrochloride	2 mg.
Water to	1,000 ml.

The ingredients are steamed to dissolve, and the pH adjusted to 3.6 approximately. The mixture is filtered, bottled, and steamed to sterilize.

The concentration of the basal medium has proved satisfactory, equal parts of double-strength medium and of vitamin B₁₂ in distilled water giving optimum growth.

Maintenance of the medium pH at about 3.6 prevents most bacterial growth, but does not prevent growth of fungi which are particularly favoured by the 30° C. temperature and long incubation. Provided all tubes are adequately heated and inoculated with care, contamination rarely occurs, although it has been troublesome in experiments without preliminary heating. Growth is good down to pH 2.5, but appears to be retarded considerably below this, and at this pH vitamin B₁₂ loses activity at room temperature (Smith, 1951).

Stock Cultures of the *Euglena*.—These were maintained for 10 months by weekly subculture in 10 ml. volumes of single-strength basal medium with 50 μg./ml. crystalline B₁₂, and at between pH 3 and pH 4. There was no development of vitamin B₁₂ independent variants, although several hundred tubes with no vitamin B₁₂ were inoculated in this time. There was, however, some change in growth characteristics after four to five months, during which period boron was not included in the medium. Growth was no longer as luxuriant and rapid as when the culture was first received, but with the addition of boric acid, growth improved, and the organism now appears to be stable in this respect. It is doubtful if this simple basal medium supplemented only by crystalline vitamin B₁₂, and kept at acid pH, can provide ideal conditions for reproduction. Cultures have also been maintained from time to time with liver extracts, as recommended by Hutner *et al.* (1949), but with no obvious difference in responsiveness to vitamin B₁₂. Cultures with 80 μg./ml. vitamin B₁₂ are now being used to increase inoculum density. Greater concentrations are not advised because of the increased carry-over of vitamin in the inoculum and because the resulting increase in density of growth leads to excessive alkali production and therefore to greater risk of bacterial contamination.

If a culture becomes contaminated it may be purified by growth on nutrient agar plates, supplemented with basal medium and vitamin B₁₂, to obtain separate colonies of the *Euglena*. Plates are incubated in the warm atmosphere of the water bath where light also assists growth.

Standards.—A crystalline vitamin B₁₂ solution, either 20 μg./ml. or 1 μg./ml., is diluted to give final concentrations of 50, 25, 15, 10, 5, 2.5, and 1.25 μg./ml. At least four tubes of each concentration are set up so that a set of seven standards requires a minimum of 28 tubes.

Stock standards certainly retain potency for one to two weeks, but show on average about a 10% loss in potency after a month at 4° C. They are, therefore, preferably prepared fresh at least every fortnight and set up together with some tubes of the previous standards for comparison.

Material for Assay

Body fluids are kept frozen until tested, and, as activity may decline on keeping, assays should be made if possible not more than three days after collection of the specimen.

As quantities of serum available for assays are usually limited, it is economical and time-saving to vary dilutions according to the probable vitamin B₁₂ concentration. All tests are preferably set up in more than one dilution with at least five tubes for each dilution, but, when large numbers of tests of sera and urines are being set up, it has been convenient to use only one dilution (e.g., 1 in 40, which allows the assay of material with between 50 and 2,000 $\mu\mu\text{g./ml.}$). When low values are expected, as with serum and urine from patients with pernicious anaemia in relapse, dilutions in the range 1 in 8 to 1 in 20 are used. Serum from patients with a slightly higher level (e.g., cases of pernicious anaemia on fortnightly or monthly maintenance injections of B₁₂ or liver and taken towards the end of the interval between injections) may require to be diluted 1 in 20 and 1 in 40. When high values are likely, as shortly after injection of vitamin B₁₂, urine and serum are set up at dilutions of 1 in 40, 1 in 400, and 1 in 4,000 or more. Liver extracts for assay require dilution to about 1 in 400,000 to 1 in 800,000.

Bacterial contamination of specimens must be avoided in view of the production of vitamin B₁₂ by some organisms and of its removal from solution by others, particularly by some strains of *Bact. coli* (Davis and Mingiolo, 1950, confirmed by Hayes and the author, unpublished data). Ternberg and Eakin (1949) found that vitamin B₁₂ in combination with a factor in gastric juice was not available to *Bact. coli*, but whether the serum factor-B₁₂ combination behaves in a similar way has not yet been investigated. The activity of some urines stored at 4° C. has in fact been found to increase, presumably either from breakdown of previously inactive compounds or from bacterial synthesis. Addition of toluene has assisted preservation, but the volatile preservatives recommended by Hutner and Bjercknes (1948) may be more suitable for this purpose, provided 100° C. heating is carried out. Urine must be completely free from any contamination with faeces because of its high content of vitamin B₁₂.

Reading and Calculating Results

Growth is read by measuring turbidity in a photoelectric colorimeter, using a red filter, and is recorded as optical density. The 4 ml. final volume

in each tube is convenient both for pipetting and for reading with the colorimeter.

Vigorous shaking is required to disperse clumps of algae, and tubes with serum froth markedly. Bubbles are dispersed immediately by the addition of one drop of a caprylic alcohol-water (1 in 5) mixture. This should not be added before vigorous shaking, because of the occasional fragmentation of algae and undue cloudiness which may result.

The more concentrated the serum, the greater the turbidity due to serum proteins. At the acid pH of the basal medium there is little tendency for precipitation even with 100° C. heating, but, as the isoelectric point of the protein is approached, turbidity increases. The pH is only very slightly altered by the addition of dilutions of serum, but with profuse growth of the *Euglena*, alkali is produced which may raise the pH above pH 5 (the pK of methyl red). This degree of alkalinity is produced by the *Euglena* in the presence of an aqueous concentration of 50 $\mu\mu\text{g.}$ per ml. of vitamin B₁₂. The alkali formed by the *Euglena* is proportional to the turbidity of the culture and therefore to the amount of vitamin B₁₂ available for growth (Fig. 1). Tubes with sera diluted 1 in

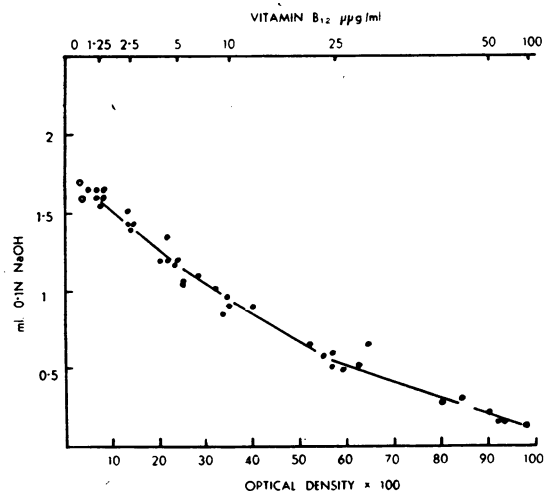


FIG. 1.—Titration of alkali produced by growth of *Euglena* in 4 ml. volumes with vitamin B₁₂ standards (1.25, 2.5, 5, 10, 25, 50, 100 $\mu\mu\text{g./ml.}$) is shown, using phenolphthalein as indicator. With 1.25 $\mu\mu\text{g./ml.}$ B₁₂ there is little growth, so that 1.6 ml. 0.1 N NaOH is required to raise pH from 3.6 to 9.7, while with 50 $\mu\mu\text{g./ml.}$ B₁₂ there is profuse growth and only 0.15 ml. 0.1 N NaOH is required. Alkali production by the *Euglena* is proportional to amount of growth and therefore to vitamin B₁₂ available.

32 or less, haemolysed sera, concentrated urines, bile, etc., may show colour or turbidity due to the test fluid itself which can be read in the supernatant after the *Euglena* cells have all sedimented,

being immobilized in the presence of caprylic alcohol. This blank reading is subtracted from the total turbidity, and the vitamin B₁₂ concentration is then calculated by comparison of this figure with those found with the standard solutions, which are plotted on log x mm. and cm. graph paper giving the type of curve shown in Fig. 2. Concentrations

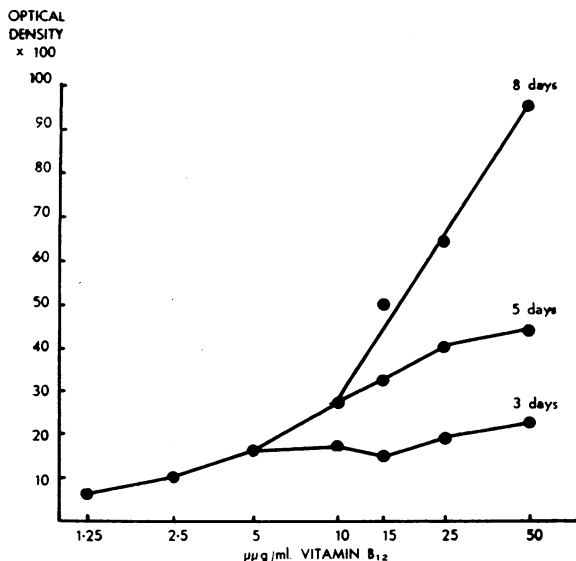


FIG. 2.—Growth of *Euglena gracilis* in presence of seven concentrations of crystalline vitamin B₁₂ showing the need of long incubation to give differentiation between growth with varied strengths of vitamin. Each point corresponds to an average reading of three tubes.

normally found in body fluids are conveniently measured in $\mu\text{g./ml.}$

Control tube readings vary from batch to batch, particularly as the result of difference in inoculum size and carry-over of unabsorbed vitamin B₁₂ in each drop of inoculum. Average optical density readings are from 0.02 to 0.06, but may be slightly higher. Standard readings also vary between batches, and therefore sets of standards must always be included.

It should be noted that incubation for as long as 10 days may be necessary to allow differentiation of growth with higher concentrations of vitamin B₁₂. Growth continues until all available vitamin B₁₂ has been used up, or until, with 150 $\mu\text{g./ml.}$ or more, growth reaches the maximum concentration of about 5 to 7 million cells per ml. (Hutner *et al.*, 1950; Robbins *et al.*, 1950b). Absorption of vitamin B₁₂ in 50 $\mu\text{g./ml.}$ concentration is rapid, and within two days of inoculation no vitamin B₁₂ is detectable in the supernatant, indicating that cells absorb much

more than their immediate requirements and continue to divide using this store. The vitamin B₁₂ requirement for the formation of each *Euglena* cell has been stated to be about 4,900 molecules (Hutner *et al.*, 1950) and about 7,100 molecules (Robbins *et al.*, 1950b). Figures of from 6,000 to 7,000 have been found by the author using varying initial concentrations of vitamin B₁₂ and taking the molecular weight as 1300. Slight variation in requirement is likely in view of the difference in cell size noted from time to time by Robbins *et al.* (1950b). Heating at 100° C. does not liberate vitamin B₁₂ from the *Euglena* cell, indicating, as expected, a radical alteration of the molecule in metabolism.

Sensitivity, Accuracy, and Specificity of the Method

Sensitivity.—While the sensitive range of assays with lactobacilli and *Bact. coli* lies above 50 $\mu\text{g./ml.}$, that of the *Euglena* method is from 5 to 25 $\mu\text{g./ml.}$ and as little as 1 $\mu\text{g./ml.}$ can be detected.

In body fluids, however, it is not practicable to detect less than about 10 $\mu\text{g./ml.}$, as serum present in greater concentration than 1 in 8 coagulates with heating at 100° C. and may be inhibitory to growth. Urine likewise may be inhibitory at 1 in 10 or 1 in 20 so that a concentration of less than 10 to 20 $\mu\text{g./ml.}$ cannot be detected. Cerebrospinal fluid, partly because of its low protein content, can be set up in higher concentration than serum; 1 in 4 has not been inhibitory. Whole blood, milk, and bile produce a turbid or coloured solution which is unsuitable for direct assay, particularly because of the resulting interference with illumination of the alga.

Accuracy.—The definite molecular vitamin requirement of each cell, already mentioned, makes the number of cells formed with sufficient time for full growth and the resulting turbidity with standard solutions of vitamin B₁₂ very consistent from batch to batch. With the more complex fluids such as serum, urine, and gastric juice, results are also consistent when assays are made on fresh material. When test fluids, for example serum and gastric juice, contain substances capable of combining with vitamin B₁₂, the extent to which the vitamin and its fractions may be reabsorbed and thus reactivated after heating will influence the true assay value unless the combining power can be destroyed. Several tests of serum and gastric juice, however, indicate that variation of time of heating at 100° C. does not greatly alter the result within the first hour, but after that the activity of the vitamin is reduced. There is a much

quicker liberation of vitamin B₁₂ when heating is carried out *after* the addition of basal medium at pH 3.6 than when heating is done at pH 7.5 *before*

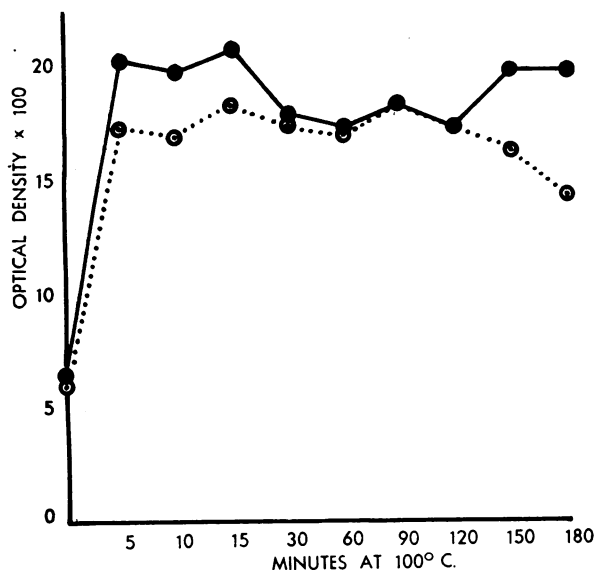


FIG. 3.—The effect of time of heating at 100° C. of normal serum (●) and of serum from a patient with pernicious anaemia during treatment with vitamin B₁₂ (○) is shown. The basal medium (pH 3.6) was added *before* heating. Release of vitamin B₁₂ is rapid—maximum at five to 15 minutes. Each point represents average reading of four tubes.

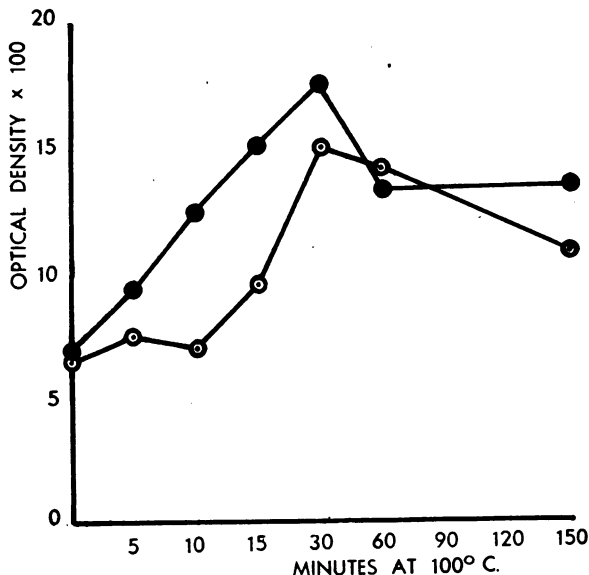


FIG. 4.—The effect of time of heating at 100° C. of normal serum (●) and of serum from a patient with pernicious anaemia during treatment with vitamin B₁₂ (○) in water at pH 7.5 is shown. The acid basal medium was added *after* heating. Release of vitamin B₁₂ is slow—maximum at 30 minutes. Each point represents average reading of four tubes.

the addition of basal medium (Figs. 3 and 4). Pooled normal serum, and pooled sera from patients with pernicious anaemia receiving maintenance doses of vitamin B₁₂, did not differ in their power of binding of vitamin B₁₂. The optimum time at 100° C. in routine assays where basal medium is added before heating is about 15 minutes. The extent of recovery of known amounts of the varieties of vitamin B₁₂ added to such fluids is usually almost complete.

Specificity.—As mentioned in the introduction, no substance has yet been identified which can replace vitamin B₁₂ in the metabolism of the *Euglena* cell. Hutner *et al.* (1949) showed that nicotinic acid, pantothenic acid, pyridoxine HCl, biotin, pteroylglutamic acid, *p*-aminobenzoic acid, and thymidine were without significant effect on the growth response, either alone or in combination with vitamin B₁₂ in the concentrations with which the tests were made. These results have been confirmed where they have been repeated as shown below and some additional substances have been tested.

Cysteine hydrochloride, nicotinic acid, pantothenic acid plus glycine, pteroylglutamic acid plus *p*-aminobenzoic acid, and methionine were added in final concentrations of 50, 5, 0.5, and 0.05 μg./ml., with and without vitamin B₁₂, 5 μg./ml. Nicotinic acid, 50 μg./ml., was completely inhibitory, while 5 μg./ml. was not. Pteroylglutamic acid, *p*-aminobenzoic acid, pyridoxine hydrochloride, glycine, pantothenic acid, and uracil in 0.5 μg./ml. concentration without vitamin B₁₂ and in 50, 5, 0.5, 0.05 μg./ml. concentrations with vitamin B₁₂ 5 μg./ml. were also tested. Ethyl alcohol, 12.5%, 2.5%, 0.25%, 0.025%, 0.0025%, and ammonium hydroxide, 5%, 0.5%, 0.05%, and 0.005%, with and without vitamin B₁₂ 5 μg./ml., were tested because of their use for dissolving some of the substances listed above. Ethyl alcohol, 12.5%, but not 2.5%, and 5% ammonium hydroxide, but not 0.5%, were inhibitory. Leucostoc citrovorum factor (folinic acid), 75 μg./ml., also did not replace vitamin B₁₂.

Although some variation in amount of growth was found with certain concentrations of some of these substances in the presence of vitamin B₁₂, there was no evidence of substitution in its absence. When pantothenic acid and glycine in final concentrations of both 250 and 25 μg./ml., alone and together, were added to normal serum diluted 1 in 40, no significant alteration in growth response was found. Both normal sera and sera from patients with pernicious anaemia, however, have about 20% greater activity when heated in

the presence of 0.05% ascorbic acid. Inclusion of ascorbic acid at the above concentration in the standard tubes containing vitamin B₁₂ in distilled water, however, has decreased growth so that fair comparison cannot be made by including ascorbic acid in all tubes of an assay batch. Ascorbic acid, 0.005%, with vitamin B₁₂ and distilled water was not inhibitory. Sulphathiazole, penicillin, streptomycin, aureomycin, chloramphenicol, and *para*-aminosalicylic acid when added in concentrations likely to be found in human serum during treatment with these drugs were also without significant effect on growth of the alga.

Comparative bioautography of vitamin B₁₂ and related factors using *Euglena gracilis* and *L. leichmannii* has shown that the *Euglena* responds only in the area of the slower moving factors where vitamin B₁₂ is expected to appear and does not respond to pure desoxyribonucleic acid or to similar substances in both liver extract and corn steep liquor which stimulate growth of the lactobacillus (Picken and Bauriedel, 1950).

A wide variety of substances has also been tested by Robbins *et al.* (1950b) using *Euglena gracilis*, mainly in plate assay, and none was found to replace vitamin B₁₂.

The only substance which has yet been claimed to have any activity in replacing vitamin B₁₂ for the *Euglena* is 1, 2, dimethyl-4, 5-diaminobenzene which may be contained within the vitamin B₁₂ molecule. Its action, however, was incomplete because of its low solubility and a concentration one half to one million times that of the equivalent amount of vitamin B₁₂ was required (Woolley, 1951).

It has not yet, however, been established that all the material in body fluids stimulating growth of the *Euglena* is haemopoietically active. In order to do this it will be necessary to assess the changes in both free and combined serum vitamin B₁₂-equivalent, in comparison with the changes in peripheral blood and bone marrow of patients with pernicious anaemia under treatment (Mollin and Ross, 1952). In spite of this reservation, preliminary results indicate that the method is of value in investigating megaloblastic anaemias and in following the absorption and excretion of the vitamin.

Concentrations in Body Fluids

Concentrations stated below as "total" indicate the amounts of vitamin B₁₂ equivalent detected after 100° C. heating for 15 minutes; as "uncombined" (free) after 56° C. heating for 30 minutes;

and as "combined," the difference between "total" and "uncombined."

The total vitamin B₁₂ equivalents found by *Euglena* assay have already been reported as from 350 to more than 1,000 μμg./ml. in normal human serum, as up to 16 μμg./ml. in cerebrospinal fluid, and as up to 200 μμg./ml. in urine, while sera from four patients with pernicious anaemia had much lower concentrations than are normally found (Ross, 1950). Further observations have extended the range of values found in both normal human serum and urine. Details of these results were given in a recent paper (Mollin and Ross, 1952).

Human ascitic and pleural fluid, and gastric juice from subjects without megaloblastic anaemia, when heated at 100° C. allowed growth comparable to that found with normal human serum. Part of the activity of the first two fluids has been combined and part free, but most specimens of gastric juice have had no uncombined activity. One specimen of knee-joint fluid and of acromial bursar fluid with high protein content had no uncombined activity and a combined equivalent of 150 μμg./ml. Human seminal fluid and plasma (oxalated, citrated, or heparinized) gave results comparable to serum.

Guinea-pig, horse, and rabbit serum have also been assayed with the *Euglena*. Samples from these three species have had variable concentrations ranging from 200 to 4,000 μμg./ml., but rabbit serum has shown particularly high concentrations. One rabbit serum had the equivalent in μμg./ml. vitamin B₁₂ of 36,000 total and 29,000 uncombined and another had 50,000 total and 40,000 uncombined.

The vitamin B₁₂ equivalents in sera then vary considerably even within species, and, in human sera on which the majority of observations has been made, this variation is mainly of the uncombined substance, while combined values remain much more constant. Combined values rarely exceed 500 μμg./ml. in normal serum, so that higher total concentrations are mainly due to free material, suggesting either that combining power of serum is limited or that the material in circulation is not readily able to be bound. Both the combined and free material, like pure vitamin B₁₂, is microbiologically inactivated by 100° C. heating for one hour at pH 12. The significance of this uncombined material is more fully discussed elsewhere (Mollin and Ross, 1952).

Fresh, 24-hour collections of urine from normal subjects contain readily detectable amounts of the

vitamin, although occasional very dilute samples are inactive. Heating of urine at 100° C. has increased the yield of vitamin by only 0 to 10%, suggesting that there is a lack of combining substance in urine.

There are as yet few reports of the concentrations of vitamin B₁₂ in body fluids. The presence of a *Euglena gracilis* vitamin in urine was originally demonstrated by Hutner (1936). *L. leichmannii* assays of whole blood gave a range of from 600 to 1,400 μμg./ml. in 10 normal human subjects and of from 400 to 15,000 μμg./ml. in 15 different animal species (Couch *et al.*, 1950). Of these animals tested, rabbit blood had the greatest amounts, although these were not so high as two of those found by *Euglena* assay and reported above. *L. leichmannii* assays of urine have also been reported. Chow *et al.* (1950) and Chow (1951) were unable to detect the vitamin in normal urine, but Girdwood (1951) has given the results of such assays on the urine of normal subjects, and of patients with sprue syndrome and with pernicious anaemia.

Summary

A method of assay of vitamin B₁₂ equivalent in body fluids using *Euglena gracilis* var. *bacillaris* is described. This microbiological method is the most sensitive available, detecting as little as 1 μμg./ml. and giving greatest accuracy in the range of from 5 to 25 μμg./ml.

Material in body fluids stimulating growth of the *Euglena* may be "combined" or "uncombined." The maximum amount of combined material is detected after 100° C. heating at pH 3.6 for 15 minutes, and the vitamin is more rapidly freed from combination in serum when heated after addition of the acid basal medium than when heated before such addition. The uncombined

vitamin is detected without such preliminary heating.

Human plasma, pleural, ascitic, seminal, bursa and joint fluid, and gastric juice assayed with the *Euglena* contained amounts similar to those found in normal human serum.

A number of substances, including amino-acids, vitamins, and antibiotics, were tested by *Euglena* assay and were not found to replace vitamin B₁₂.

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