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An automated microbiological method for the measurement of vitamin B_{12}

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SYNOPSIS An automated method for the microbiological assay of vitamin B_{12} is described. A chloramphenicol-resistant strain of *Lactobacillus leichmannii* is used as the test organism and this eliminates the need for sterilization or aseptic addition. Precipitation of the serum protein is avoided by dilution of the serum with a glutamic/malic acid solution before heating the preparation to free the vitamin B_{12}. Tests can be set up at a rate of 80 an hour and after incubation they can be read at 160 an hour. By interfacing a programmed electronic calculator directly with the Mecolab M which is used for the assays, results are printed directly in ng/l.

The microbiological assay of vitamin B_{12} in serum was first described by Ross (1950) more than 20 years ago using *Euglena gracilis var. bacillaris* as the test organism. The technique was tedious and time consuming and required elaborate procedures for the cleaning of glassware. Later the Z strain of *Euglena gracilis* was introduced and this shortened the growth period required for the assay to five days (Hutner, Bach, and Ross, 1956). This technique gave highly reproducible results and appeared specific for vitamin B_{12} in serum. Spray (1955) described a rapid assay technique using *Lactobacillus leichmannii* as the test organism, the method gave rather higher results than were obtained using *Euglena* and was not as specific but had the advantage of being able to provide a result in 24 hours. Variations of this basic method are used by many laboratories. In 1965 Lau, Gottlieb, Wasserman, and Herbert described an assay technique based on isotope dilution. This technique is useful for laboratories making a limited number of measurements, but it has not been used where very large numbers of samples require measurement, and more recently doubts have been cast on the validity of some of the results (Raven, Robson, Morgan, and Hoffbrand, 1972).

We describe here a fully automated method for the microbiological measurement of vitamin B_{12} using *Lactobacillus leichmanni* as the test organism. Results are available in 24 hours and the equipment can set up tests at a speed of 80 an hour and can read results following incubation at 160 an hour.

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Materials and Methods

A chloramphenicol-resistant strain of *L. leichmannii* was developed from the standard strain (ATCC 7830 NCIB 8117) by a method similar to that described by Davis, Nicol, and Kelly (1970) for *Lactobacillus casei*. The standard strain of the organism was passaged through liquid media containing increasing concentrations of chloramphenicol. A stock solution of 0·1% chloramphenicol base in 1% ethanol was prepared. Increasing concentrations of the antibiotic were added to one ounce screw-capped bottles containing 10 ml of single strength assay media with added vitamin B_{12} (50 ng/l). Each concentration was prepared in duplicate and chloramphenicol was added in the following increments: by 0·5 mg steps from 1 to 10 mg/l; then in 1·0 mg steps to 20 mg/l; 5 mg steps to 100 mg/l; and thereafter 10 mg steps to 300 mg/l. In the event of poor growth at any stage the organism was subcultured back into the previous concentration and incubated until satisfactory growth was achieved. The organism was suitable for use in the assay when tolerant to a chloramphenicol concentration of 200 mg/l. In the assay proper chloramphenicol was used at a concentration of 10 mg/l. The differential between the two concentrations of antibiotic was necessary to ensure vigorous growth of the test organism.

PRESERVATION OF THE TEST ORGANISM

The chloramphenicol-resistant organism was grown in maintenance media without chloramphenicol until a heavy growth was obtained; this usually took 24-36

hours. The culture was then centrifuged and the supernatant discarded. Five per cent sodium glutamate was added to give a heavy suspension of the organism. A few drops of this suspension were then added to an ampoule containing about 100 small sterile ceramic beads. The ampoule was gently shaken to coat the beads with the organism, it was then connected to a vacuum system and the organisms dried from the liquid phase. The dried organisms were stored in a screw-capped bottle to which two crystals of indicator silica gel had been added. The method was essentially that described by Annear (1962).

PREPARATION OF INOCULUM

On the day before the test a tube containing 10 ml of maintenance media was inoculated with one ceramic bead containing the dried organism. The use of assay media with added vitamin B_{12} for the preparation of the inoculum rather than less well defined media such as broth had the advantage of promoting the growth of the test organisms under conditions similar to those found in the test proper. The culture was incubated overnight at 37°C after which it was centrifuged and the supernatant discarded. The organisms were suspended in saline to give an optical density of 1·0. To each litre of double-strength assay medium 2·0 ml of this suspension was added immediately before the test.

SUBSTRATE

Double-strength assay medium was prepared according to the protocol in table I. After mixing the pH was adjusted to 6·9, the medium was then stored at −20°C in 2-litre plastic containers. It will keep under these conditions for many months. Alternatively Difco B_{12} assay medium USP may be used provided the pH is first adjusted to 6·9. Immediately before use chloramphenicol was added to a concentration of 20 mg/l.

MAINTENANCE MEDIUM

This was single-strength assay medium containing 50 ng/l of vitamin B_{12} and 100 mg/l chloramphenicol.

DISASSOCIATING VITAMIN B_{12} FROM ITS CARRIER

Most of the vitamin B_{12} present in serum is bound to a protein transcobalamin and is not available to the test organism in this form. The vitamin is usually freed from the protein carrier with the aid of heat; this also results in precipitation of the serum proteins unless the preparation has a low pH. When using *E. gracilis* as the test organism precipitation of the serum proteins does not occur because the pH of the preparation is 3·6, whereas assays using *L. leichmannii* are run at a pH of 5·9 and heating will precipitate the proteins. To overcome this problem serum samples were diluted into 2·45 ml of a solution containing 0·2 g of malic acid and 3·0 g of glutamic acid per litre; the pH of this solution should be 3·6. The tubes were then placed in a water bath at 100°C for five minutes after which they were cooled and processing continued. The optimum heating time was determined by placing an acidified sample in a boiling water bath and removing aliquots at one-minute intervals. No increase in free vitamin B_{12} occurred after three minutes' heating.

STANDARDS

A stock solution was prepared by dissolving 10 mg of crystalline cyanocobalamin (Sigma) in 500 ml of distilled water to give a concentration of 20 mg/l. One millilitre of this solution was then diluted to 100 ml with distilled water to give a concentration of 200 μg/l. A working solution was prepared by taking 1 ml of the last dilution and again diluting to 200 ml with distilled water. The working solution may be kept for four weeks when stored at 4°C; the stock solution will keep for months if refrigerated. The working

Enzymatic casein hydrolysate (NBC)	200 ml	Ca pantothenate	2 mg
Glucose	40 g	Pyridoxine HCl	4 mg
Sodium acetate (anhydrous)	12 g	Thiamine HCl	4 mg
L-Cystine	200 mg	Nictoninic acid	2 mg
DL-Tryptophan	400 mg	Biotin	10 μg
Adenine sulphate[1]	10 mg	Folic acid	1 mg
Guanine HCl[1]	10 mg	Pyridoxal	1 mg
Uracil[1]	10 mg	p-Amino benzoic acid	1 mg
Xanthine[1]	10 mg	DL-α-alanine	1 g
KH_2PO_4	1 g	Thiomalic acid	1 g
K_2HPO_4	1 g	$CaCl_2$	200 mg
$MgSO_4$	400 mg	Guanosine	200 mg
NaCl	20 mg	Guanylic acid	200 mg
$FeSO_4.7H_2O$	20 mg	Cysteine	1 g
$MnSO_4.7H_2O$	20 mg	Glutamine	400 mg
Tween 80/polysorbate 80	2 g	Distilled water to	1 litre
Riboflavin	2 mg		

Table I *Double-strength assay medium*

Dissolved by suspending in a small volume of H_2O and then adding concentrated KOH drop by drop until solution is complete.

Standard Tube Number	Volume of Working Solution	Volume of H_2O	Concentration in ng/l	Concentration in ng/l after Dilution with Substrate
0	0	4·0	0	0
1	0·4	3·6	100	1·0
2	0·6	3·4	150	1·5
3	0·8	3·2	200	2·0
4	1·0	3·0	250	2·5
5	1·2	2·8	300	3·0
6	1·6	2·4	400	4·0
7	2·0	2·0	500	5·0
8	2·4	1·6	600	6·0
9	3·2	0·8	800	8·0
10	4·0	0	1000	10·0

Table II *Preparation of working solutions for standard curve*

solution was used to prepare the final standard solutions as shown in the protocol (table II) and these were sampled in the same manner as the serum or other material to be assayed.

ASSAY PROCEDURE

The method was designed to use a specifically modified version of the Joyce Loebl automated chemistry apparatus, Mecolab M (fig. 1). This unit provides the following automated facilities—sample dilution, reagent addition, mixing of bacterial growth after incubation, measurement, and digital estimation of bacterial growth.

A diluter on the universal sampler unit was adjusted to sample 0·05 ml of serum and to dilute this with 2·45 ml of malic/glutamic acid solution. The sampler holds 40 serum samples and 40 assay tubes in circular racks. The machine was programmed to sample each serum and deliver it with 2·45 ml of malic/glutamic acid solution into a plastic disposable assay tube. When this has been completed the machine stops automatically. Racks of assay tubes were removed and placed in a water bath at 100°C for five minutes, allowed to cool, then returned to the turntable.

Double-strength assay medium was inoculated with the test organism as described earlier and the flask placed on a magnetic stirrer on top of the sampler unit. A diluter was used to deliver 2·5 ml of the inoculated medium into each of the tubes giving a final dilution of 1 in 100. Standards were treated in the same manner as the serum samples. They were set up in triplicate with two extra blanks and two extra top standards. After setting up, racks of tubes were incubated at 37°C for approximately 24 hours or until the top standard reached an optical density

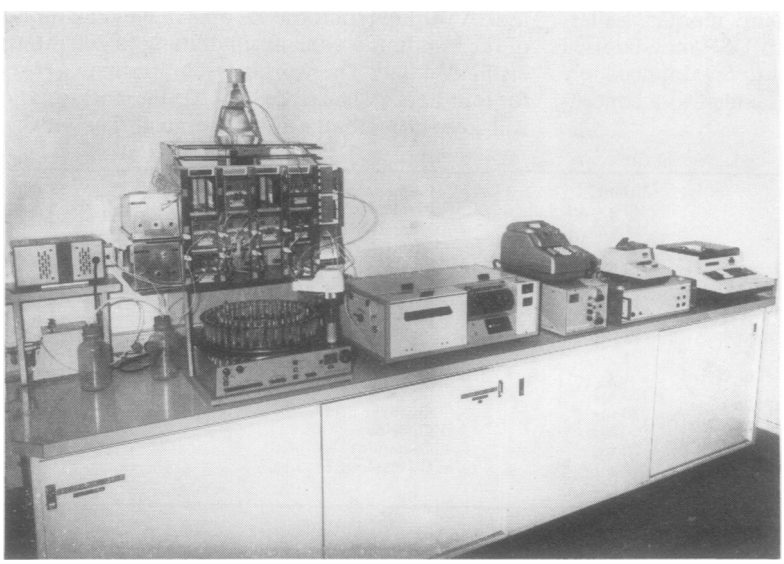

Fig 1 *Mecolab M showing from left to right sample preparation unit, auto-colorimeter, A/D converter and printer, interfaced calculator with tape reader for insertion of programs.*

of 0·33. The racks were then returned to the Mecolab where the contents of each tube were mixed and the growth measured in the colorimeter. Results were recorded in digital form onto paper tape.

CALCULATION OF RESULTS

Readings obtained from the standards can be used to plot a curve on linear graph paper, and the concentration of the unknown can be determined by interpolation. However, this is inconvenient when large numbers of samples are being processed. A more satisfactory approach is to use a programmable electronic calculator such as the Diehl Combitron S with punch tape reader. The program can be punched onto paper tape and by passing this through the tape reader the machine can be rapidly set up.

The Combitron S uses the following formula for linear interpolation:

$$y = d - \frac{(d - b)(c - x)}{(c - a)}$$

The formula assumes a straight line between any two points on the curve and this introduces a small degree of error which has not been found to exceed 2·5%.

The program was fed into the punched tape reader and the absorbence values obtained from the standards were entered into the Diehl Combitron S where they were matched with the standard concentrations that they represent, ie, 100, 200, 300, 600, and 1000 ng/l. The unknown values were then entered into the machine and a number of logical decisions were made to determine between which two points on the curve the unknown fitted. The machine then applied the linear interpolation formula using the appropriate values for A, C, B, and D, and the result is printed together with the original absorbence value onto paper tape.

Example (fig 2)

$x = 267$

$b = 300$

$a = 200$

$d = 600$

$c = 400$

$$y = d - \frac{(d - b)(c - x)}{c - a}$$

$$= 600 - \frac{(600 - 300)(400 - 267)}{400 - 200}$$

$$= 600 - \frac{(300)(133)}{200}$$

$$= 600 - 199{\cdot}5$$

$$= 400{\cdot}5 \text{ ng/l}$$

When using small calculators in this way the time taken for each calculation extends over several seconds and this must not be allowed to exceed the time taken by the Mecolab to measure sample absorbence. A maximum of 20 seconds is permitted and this restricts the number of standards that can be used.

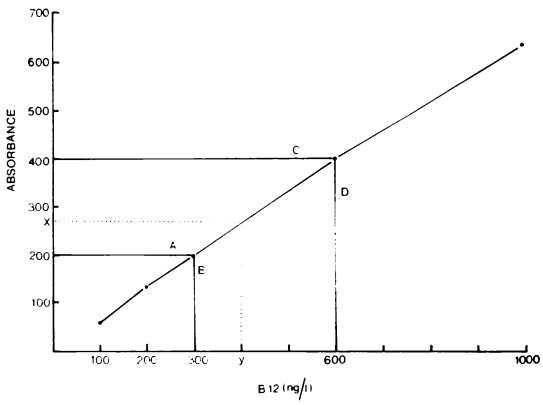

Fig 2 *Point selection for linear interpolation.*

The output of the Mecolab may be interfaced directly with the Combitron S; the calculator will then automatically print the absorbence value and the concentration in ng/l.

Vitamin B_{12} was also measured using *Euglena gracilis* as the test organism (Nicholas and Pitney, 1958). The normal range for this laboratory is 160-875 ng/l.

A total of 150 sera was assayed by both the automated *L. leichmannii* and *E. gracilis* methods. One hundred were from normal individuals and 50 were from patients with a variety of disorders which included vitamin B_{12} malabsorption and liver disease.

Results

The comparison between the *L. leichmannii* and *E. gracilis* assays are shown in fig 3 and the difference was statistically significant ($0{\cdot}001 < P < 0{\cdot}01$). However, the difference was constant at all levels. Results using the present method if multiplied by a factor of 0·734 gave results similar to those obtained using *Euglena* and results using *Euglena* if multiplied by a factor of 1·3 gave results similar to those obtained with *L. leichmannii*; using these factors the correlation coefficient was 0·951. The least significant difference using an analysis of duplicates with *L. leichmannii* was 108 ng/l with a coefficient of variation of 9·445%; this was not quite as good as that obtained with the *Euglena* assay of 82·9 ng/l and a coefficient of

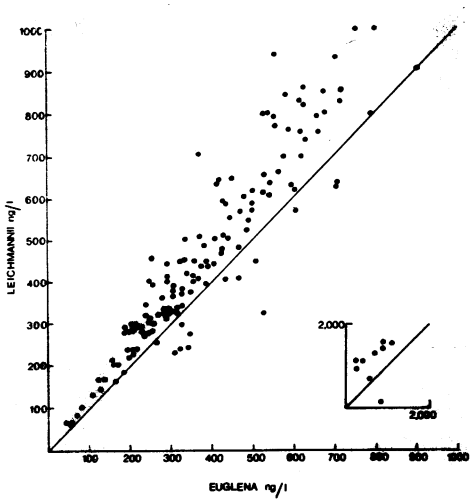

Fig 3 *Comparison of vitamin B_{12} values obtained from 150 sera by* E. gracilis *and* L. leichmannii *assays.*

variation of 7·553%. The mean of the 100 sera from normal individuals was 410 ng/l (range 150-1000 ng/l) when assayed using *L. leichmannii*. Ten serum samples had a low vitamin B_{12} concentration when assayed using *L. leichmannii* as the test organism. The assay was repeated using *E. gracilis* as the test organism and the results are shown in table III.

A number of workers have recommended the addition of cyanide to the assay system (Girdwood, 1960; Brandt and Metz, 1961; Spray, 1962; Matthews, 1962) since it has been suggested that *L. leichmannii* is only sensitive to cyanocobalamin. To determine the effect of cyanide on the assay 32 serum samples were assayed with and without the addition of sodium cyanide (0·0125 g/l); the results showed no significant difference, Z = 0·02941.

Euglena gracilis n g/l)	*Lactobacillus leichmannii* (ng/l)
(36	65
50	60
50	54
72	100
120	140
60	80
165	200
130	165
100	130
180	180

Table III *Comparison of results obtained in 10 samples with low levels using* Euglena gracilis *and* Lactobacillus leichmannii.

Discussion

A rapid, fully automated method suitable for the measurement of serum vitamin B_{12} in serum has been described. The method uses a chloramphenicol-resistant strain of *L. leichmannii* as the test organism and this has removed the need for sterilization or aseptic addition.

Precipitation of serum protein has been overcome by the use of a malic/glutamic acid diluent before heating the samples to free the vitamin from its protein carrier. When combined with an automated folate assay system (Davis *et al*, 1970) a large number of samples can be processed and results from both assays are available within 24 hours.

The mean level for 100 sera was 410 ng/l (range 150-1000) compared with a mean of 471 (range 155-1075 ng/l) reported by Raven and his colleagues (1972). Although results are higher than those obtained with the *Euglena* assay the difference is constant and adjusted results have a close correlation.

The acid diluent is of low ionic strength and the pH of the assay medium is adjusted so that a final pH of 5·9 is achieved.

The method is particularly well suited for institutional and population surveys. Results below 200 ng/l may be confirmed using the *Euglena* assay.

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