Rapid microbiological assay of serum vitamin B₁₂ by electronic counter

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SYNOPSIS A new method of measuring the growth of *Lactobacillus leichmannii* is reported. Its adoption for the estimation of serum vitamin B₁₂ levels shortens the incubation period required to five hours at 45°C. The method is compared statistically with a standard method of estimation requiring incubation at 37°C., by duplicate determinations on 106 hospital patients. The significance of the apparently decreased accuracy of the new method at low serum levels is discussed, and re-appraisal of the optimum growth temperature of *Lactobacillus leichmannii* suggested.

The estimation of vitamin B₁₂ levels in human serum is commonly required in the investigation of megaloblastic anaemia. The chemical methods available remain insufficiently specific or are too complicated for routine hospital laboratory use. Microbiological assay is accepted as the method of choice and its value and limitations are well known (Spray and Witts, 1958; Girdwood, 1960). The choice of organism used is of necessity a compromise, but usually falls between *Euglena gracilis* and *Lactobacillus leichmannii*.

Assays using the Z strain of *Euglena gracilis* have the advantages of high specificity and high sensitivity, and require no extraction process with cyanide. An incubation period of five days is commonly required, however, and despite the use of optimal conditions of medium, light, inoculum and growth, the shortest incubation period quoted in the literature is three to four days (Anderson, 1964). For the clinician the value of this technique is thus limited. *Lactobacillus leichmannii* is slightly less specific and less sensitive for vitamin B₁₂ but is useful for routine clinical use because of the relatively short incubation period required. The length of this is mainly dependent upon the method of measurement of growth at the end of it, and to date these methods have been relatively crude—measurement of acid production, pH change, or turbidity. The turbidimetric method is generally accepted as the most convenient but usually requires an incubation period of 18 to 24 hours at 37°C., 16 hours being the shortest period quoted (Girdwood, 1954).

The introduction of electronic particle counters

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has allowed accurate and rapid determination of cell counts in blood, and also of bacterial counts (Swanton, Curby, and Lind, 1962) in various media. The use of such a counter is described in this paper in an attempt to refine measurements of growth and to reduce the long period of incubation required before measurable growth can be accurately recorded. A method is described for the estimation of vitamin B₁₂ levels in human serum using an incubation period of five hours after inoculation with *Lactobacillus leichmannii*. The method is compared with one of the standard techniques (Matthews, 1962) using this organism, its accuracy being determined by duplicate determinations, by both methods on 106 sera from hospital patients. The results are subjected to statistical analysis.

METHODS

STANDARD METHOD One hundred and six sera referred to the laboratory for estimation of serum vitamin B₁₂ levels were assayed in routine batches using the technique as described by Matthews (1962) with minor modifications only. Samples of venous blood, each of 20 ml., were supplied and the serum separated and used either immediately, or after storage at -20°C. The volume of serum used for each assay was 2.5 ml., and each assay was repeated once within the same week using a different standard.

NEW METHOD Further samples of the same sera, 0.5 ml. per assay, were removed and assayed by the technique described below, a duplicate determination being performed as above.

Apparatus The counter was the Coulter counter, model A using a 30 micron orifice. Assays were performed

in $3 \times \frac{7}{8}$ in. pyrex test tubes with aluminium caps. Incubation was carried out in a 45°C. closed waterbath with stirrer, but without agitation of the tubes.

Organism and media The organism used was *Lactobacillus leichmannii*, National Collection of Industrial Bacteria, catalogue number NCIB 8118, which was subcultured daily in alternate broth and stab cultures and incubated at 37°C. The media used were Bacto Micro Assay Culture Agar and Bacto B₁₂ Assay Medium USP (Difco Laboratories; Baird and Tatlock, U.K.). Following rehydration of the medium in accordance with the manufacturer's instructions, the solution was gravity filtered through an 0.45 micron millipore filter.

Method of counting All specimens were counted in duplicate using a 30 micron aperture with the counter set at gain 6, ACS 5 and threshold 15. These settings were arrived at experimentally. Serial counts were performed on the same specimen at progressively increasing threshold settings until loss of count occurred. The development of a plateau suggested that all organisms were being counted and a threshold of 15 was chosen as being the most suitable to achieve this consistently and also to eliminate electrical interference. The higher counts obtained following incubation necessitated a 1 in 21 dilution in saline.

Preparation of inoculum A careful aseptic technique was developed to prevent contamination. A 24-hour broth culture of *Lactobacillus leichmannii* was centrifuged at 1,000 r.p.m. for 10 minutes in a sterile Universal container, the resulting deposit being washed in approximately 20 ml. of Evans sodium chloride injection BP (0.9%). The procedure was repeated twice, the organisms being finally suspended in approximately 20 ml. sterile saline. A background count was performed on the saline. A 1/100 dilution of the suspension of *Lactobacilli* was then mixed and counted, and the background count subtracted. The suspension was further diluted with sterile saline to make up an inoculum of 4,000,000 organisms per ml., the total volume depending on the number of assays contemplated.

Preparation of standards Cyanocobalamin standards were made up freshly for each assay and preservatives such as phenol were therefore not required. A working solution made up with distilled water to a final concentration of 100 µg. cyanocobalamin was used. Three standards, each comprising seven tubes, were made up in triplicate as in Table I.

TABLE I
PREPARATION OF STANDARD

Tube	1	2	3	4	5	6	7
Medium (ml.)	1.25	1.0	1.0	1.0	1.0	1.0	1.0
B ₁₂ standard (ml.)	—	—	0.05	0.1	0.2	0.3	0.4
Distilled water (ml.)	1.0	1.0	0.95	0.9	0.8	0.7	0.6

Mix, cap, and sterilize at 15 lb. per square inch for five minutes.

Preparation of serum One drop of a 1% solution of potassium cyanide was added to 0.5 ml. serum in a clean test tube, capped, and allowed to stand at room temperature for 45 minutes. Then 4.0 ml. of distilled water and 0.5 ml. of 0.2M sodium acetate buffer, pH 4.6, were added and the mixture steamed gently for 30 minutes.

Any adherent protein was loosened from the sides of the tube which was then centrifuged at 500 r.p.m. for one minute, followed by 2,000 r.p.m. for 10 minutes. Approximately 3 ml. of supernatant fluid was carefully removed with a clean Pasteur pipette into a further test tube and its pH adjusted to 6.8 by means of N/10 NaOH using narrow range pH paper. From each specimen, tubes were made up in groups of three (see Table II), in duplicate, for both assay and blank purposes.

TABLE II

PREPARATION OF SERUM

Tube	1	2	3
Medium (ml.)	1.0	1.0	1.0
Serum (ml.)	0.25	0.5	0.75
Distilled water (ml.)	0.75	0.5	0.25

Mix, cap, and sterilize at 15 lb. per square inch for five minutes.

Method of inoculation All tubes except tube 1 of the standard were inoculated with 0.25 ml. of the prepared inoculum (*i.e.*, an inoculum of 1,000,000 organisms per tube), and all tubes were then incubated at 45°C. for exactly five hours.

Method of calculation The value obtained for tube 1 of the standard was subtracted from the value obtained from each of the other standard tubes, and a standard growth curve was compiled by plotting vitamin B₁₂ concentration against bacterial count. The cyanocobalamin content of the serum extracts was then read in the usual way.

RESULTS

The results obtained by each method for the 106 patients in the experiment are divided into three broad groups based on the serum vitamin B₁₂ levels obtained as follows: (1) Below 170 µg./ml., (2) between 170 and 500 µg./ml., and (3) 500 µg./ml. or more (Table III). These serum levels were chosen because the normal range for the standard method in this laboratory is 170-1,000 µg./ml.

TABLE III

DISTRIBUTION OF SERA ACCORDING TO B₁₂ ESTIMATIONS BY STANDARD AND NEW METHOD (MEAN OF DUPLICATE READINGS FOR SERA OF 106 PATIENTS)¹

Estimated Vitamin B ₁₂ Level (µg./ml.)	Standard Method	Dilution Level of New Method		
		0.25 ml.	0.50 ml.	0.75 ml.
Under 170	24	29	33	31
170 to 500	37	60	52	52
500 or more	45	17	21	20
				3 ²
Total	106	106	106	106

¹There were nine cases with only one reading; five of these were very low (<170 µg./ml.). One was in the 0.25 and one in the 0.50, and three were in the 0.75 dilution group; two of the remaining three cases were in the middle of the 170-500 µg./ml. grouping in the 0.75 ml. group; and the last case in the 0.75 ml. dilution group was over 500 µg./ml.

²Classification into these broad groups was not possible for three patients with duplicate readings in µg./ml. as follows: (420, more than 500), (360, more than 500), (314, more than 500).

The means of the duplicate readings were used for this classification. Three patients had duplicate readings with open-ended values one on either side of one of the group boundaries, and they are not included in this comparison.

The new method did not give such high readings as the standard method. At all three serum dilutions, the new method grouped more patients as '170 $\mu\text{g.}/\text{ml.}$ or less' or '170 to 500 $\mu\text{g.}/\text{ml.}$ ', and fewer patients as '500 $\mu\text{g.}/\text{ml.}$ or more'.

Direct comparison of the new and standard methods was made by cross tabulation in these three broad groupings (Table IV). The new method only gave higher readings than the standard method for, at the most, five patients at the 0.25 ml. serum dilution. It did, however, show lower readings for 33 to 38 patients. With this difference in range of scale of the two methods, complete agreement between them on the broad classification of patients was inevitably low: between 63 and 67 of the 106 samples, depending on the serum dilution of the new method.

TABLE IV

AGREEMENT ON CLASSIFICATION OF SERUM VITAMIN B_{12} LEVELS ACCORDING TO STANDARD AND NEW METHOD IN THREE BROAD GROUPS (MEAN OF DUPLICATE)¹

Agreement with Standard Estimates	Dilution Level of New Method		
	0.25 ml.	0.50 ml.	0.75 ml.
Standard and new in same group	63	67	67
New estimate in higher group than standard	5	3	3
Standard estimate in higher group than new	38	36	33
Total number of cases	106	106	106

¹See note to Table III.

EXPERIMENTAL ERROR The existence of open-ended values, e.g., '500 $\mu\text{g.}/\text{ml.}$ or more', prevents a full statistical analysis of the experimental data. A crude indication of experimental error is first made for nearly all the patients in the study by cross-classification of the duplicate readings according to the three broad groups already used. Nine individual readings were not available because of errors in procedure. The number of times that these duplicate determinations were in disagreement on the broad classification of the sample is shown as a percentage of the total number of samples with duplicate readings by each method (Table V). The percentage disagreement varied between 21 and 26. The differences in these percentage disagreements between the standard and the new method were not statistically significant ($p > 0.1$) neither were the differences between the three serum dilutions of the new method ($p > 0.1$).

TABLE V

EXPERIMENTAL ERROR OF STANDARD METHOD AND OF NEW METHOD AT THREE SERUM DILUTIONS AND DISAGREEMENT BETWEEN DUPLICATE READINGS ON CLASSIFICATION OF PATIENTS INTO THREE BROAD B_{12} GROUPS

Method of Estimation	Number of Patients with Duplicate Estimations ¹	Patients with Duplicate Readings in Disagreement on Broad Classification
Standard	106	22 (20.8%)
0.25 Serum dilution	105	24 (22.9%)
0.50 Serum dilution	105	27 (25.7%)
0.75 Serum dilution	99	26 (26.3%)

¹See note to Table III.

ANALYSIS OF VARIANCE: EXPERIMENTAL ERROR IN PATIENTS The 'analysis of variance', which permits the statistical assessment of experimental error and the testing of significance of differences in mean estimates of B_{12} , can only be made for those patients with complete sets of observations without 'open-ended' values, in this case 66 in all. In order to examine more precisely the experimental error of the two methods at different levels of vitamin B_{12} , these 66 patients with complete duplicate readings were first put into the three broad B_{12} groups already used.

The means of the duplicates of the standard estimations were used for this purpose so as to enable evaluation of error at familiar critical levels.

The experimental error of each method within each of the three B_{12} groups is based on the residual variation after eliminating the variation in B_{12} values due to differences between patients. The effect of interaction between patients and method was also eliminated, since there was the possibility that sera from particular patients might have given rise to specially high or low B_{12} estimates for one method rather than the other.

The experimental error (residual variation) is stated in absolute terms in $\mu\text{g.}/\text{ml.}$ and also in relative terms as a coefficient of variation obtained by expressing the standard deviation (residual variation) as a percentage of the corresponding mean estimate of B_{12} . This measure is more relevant since it has already been shown that the range of the estimations of B_{12} by the new method was smaller than that by the standard method. The practical importance of the experimental error is therefore shown by the coefficients of variation referred to subsequently as relative error.

There were no significant differences in experimental error of the estimation between the different serum dilutions of the new method (Table VI) in any of the three broad B_{12} groups.

It is therefore possible to pool the information for

TABLE VI

DIFFERENCES IN EXPERIMENTAL ERROR IN B₁₂ ESTIMATION BY THE NEW METHOD ACCORDING TO
SERUM DILUTION AND THREE BROAD B₁₂ LEVELS

<i>B₁₂ Level of Serum Grouped According to Standard Estimate</i>	<i>Number of Patients</i>	<i>Serum Dilution</i>	<i>Mean Estimate (B₁₂ values in µµg./ml.)</i>	<i>Experimental Error (residual standard deviation)</i>	<i>Relative Error (coefficient of variation) S.D. % Mean %</i>	<i>Homogeneity of Variance (Bartlett's test)</i>
µµg./ml.						
Less than 170	16	0.25	96.9	27.9	28.8	χ ² = 2.645
		0.50	93.4	30.1	32.2	d.f. = 2
		0.75	107.8	40.9	37.9	0.20 < p < 0.30
170-500	31	0.25	233.1	65.3	28.0	χ ² = 0.0633
		0.50	231.8	65.9	28.4	d.f. = 2
		0.75	241.4	63.9	26.5	0.950 < p < 0.975
500 or more	19	0.25	361.1	65.2	18.0	χ ² = 0.4831
		0.50	345.0	76.4	22.1	d.f. = 2
		0.75	351.6	73.1	20.8	0.70 < p < 0.80

TABLE VII

DIFFERENCES IN EXPERIMENTAL ERROR IN B₁₂ ESTIMATION BETWEEN STANDARD METHOD
(DUPLICATES) AND NEW METHOD (DUPLICATES OF THREE SERUM DILUTION COMBINED)

<i>B₁₂ Level of Serum Grouped According to Standard Estimate</i>	<i>Number of Patients</i>	<i>Method</i>	<i>Mean Estimate (B₁₂ values in µµg./ml.)</i>	<i>Experimental Error (residual standard deviation)</i>	<i>Relative Error (coefficient of variation) S.D. % Mean %</i>
µµg./ml.					
Under 170	16	Standard	100.1	18.9	18.9
		New	99.4	33.5	33.5
170-500	31	Standard	322.4	80.6	25.0
		New	235.4	65.0	27.6
500 or more	19	Standard	635.1	143.3	22.6
		New	352.3	71.1	20.3

each serum dilution to provide a single estimate of the error of the new method for comparison with the standard (Table VII). The relative error of the standard method was approximately the same in each of the three broad B₁₂ groups, varying only between 19 and 25%. The relative error of the new method varied with the B₁₂ level. It was higher in the two lower B₁₂ groups than in the highest group. For example, in the B₁₂ group below 170 µµg./ml. the coefficient of variation was 33.7% compared with 20.3% for values of B₁₂ of 500 µµg./ml. or more.

The present series was selected to provide a wide range of B₁₂ values, and no attempt has therefore been made to establish a normal range of values for the new method. The new method gives values on a shorter scale than the standard, giving much lower values at the high levels and approximately the same values as the standard at the lower B₁₂ levels.

DISCUSSION

A number of modifications were considered before the above method was adopted. In particular, although chain formation was not thought to be a problem from direct microscopic examination of the culture, the effects of variable periods (five to 20

minutes) of ultrasonic vibration, both before and after incubation, was noted. No significant change in count was found.

A four-hour incubation period was found to provide adequate growth for the production of a reliable standard curve in the majority of cases, but an occasional culture failed to provide optimal growth, regardless of the size of the inoculum. This latter problem was not found with the five-hour incubation period and its adoption is recommended.

An inoculum size of 1,000,000 organisms per tube provided the ideal amount of growth during this five-hour period. Inocula of less than 500,000 organisms per tube provided insufficient growth, while inocula greater than 2,000,000 organisms per tube produced such high final counts that considerable dilution was required to bring the count within the accuracy range of the counter, without having to recourse to correction for coincidence.

The use of the 30 micron orifice, as compared with the 100 micron aperture used in red and white cell counting, inevitably results in a greater number of orifice blockages during counting. Filtration of the medium was required to minimize this, but other solutions were used unfiltered. The Evans solution of sodium chloride for injection provided the lowest background count of numerous solutions tried

although at that time Polyfusor (Boots Pure Drug Co. Ltd.) particle-free normal saline was not available and may prove superior. Reasonable precautions to exclude dirt from all containers were taken and all were capped when not in use.

The use of filtration to remove the protein precipitate during the process of extraction of vitamin B₁₂ from serum is advocated (Matthews, 1962), but the procedure in this laboratory has been to centrifuge and decant the supernatant, and this was retained. Although Matthews (1962) has shown that negligible amounts of vitamin B₁₂ are recoverable from the precipitate, provided that cyanide extraction is used, the presence of fragments of coagulated protein in subsequent tubes will encourage blockage of the 30 micron aperture and for this reason should be guarded against.

The cultural characteristics of *Lactobacillus leichmannii*, despite its widespread use in microbiological assay, have been the subject of controversy. In Bergey's manual (Breed, Murray, and Smith, 1957) it is classified as a member of a low temperature homofermentative group. Since then it has been reclassified (Rogosa and Sharpe, 1959) as a thermobacterium, with relatively high optimum and minimum growth temperatures, and a maximum growth temperature generally in the range 45° to 50°C. Briggs (1953) also showed it to have thermophilic properties with growth at 48°C. and a heat survival time of 30 minutes at 65°C.

The influence of the temperature of the water bath on the bacterial count after five hours' incubation was therefore studied at the levels 37°C., 41°C., 45°C., and 51°C. Optimum growth occurred at 45°C. and this temperature was adopted for the remainder of the estimations. It was also noted that growth after 18 hours' incubation using the standard method was considerably greater at 45°C. than at 37°C., although the reliability of this fact was not studied. There would thus appear to be justification for further comparison of assays using a variety of methods and strains of *Lactobacillus leichmannii* at these temperatures.

Lactobacillus leichmannii will grow aerobically on subculture but is inhibited by excess aeration. It was confirmed that growth was reduced when the tubes were agitated during their five-hour period of incubation.

Statistical analysis of the results is disappointing in that the analysis of experimental error on the

subgroup of sera with complete data and no open-ended values did not reveal any increased accuracy by the new method. In fact at serum levels below 170 µg./ml. its accuracy was less than the standard and less than its accuracy at higher B₁₂ levels. This is anomalous since one would expect error to be proportional to mean B₁₂ level as it is with the standard method. This result may possibly be due to flow factors affecting the counter at lower particle concentrations. There is therefore a clear need for further estimations of B₁₂ by the new method among patients with values bordering on 170 µg./ml.

The experimental error did not appear to be related to the relative serum dilutions. There seems, therefore, little point in using all three dilutions and the 0.25 ml. serum dilution is recommended for use since it covers the greatest range (50 µg./ml. to 1,000 µg./ml.).

The new method thus appears to offer the advantages of a short incubation period (five hours) plus the need for a smaller donation of blood from the patient. At present it appears to be less accurate at low serum B₁₂ levels, but this requires further investigation. It is hoped to automate further the pipetting and counting procedures to make full use of the short incubation period and allow the assay to be completed within normal laboratory hours in one day. It is also hoped that the technique will be applicable to other microbiological techniques, particularly folic acid estimation.

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