THE MICROBIOLOGICAL ASSAY OF VITAMIN B_{12} IN LIVER EXTRACTS

I. Experimental Design and Problems of Validity

BY F. A. ROBINSON, M. E. H. FITZGERALD and J. J. GRIMSHAW
From the Research Division, Allen & Hanburys Ltd., Ware

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INTRODUCTION

For many years these laboratories have been concerned with the production of liver extracts and with the estimation of their cobalamin contents. This paper deals with the experimental methods employed, with the development of the present experimental design, and with some of the problems of validity encountered. In a later publication it is hoped to discuss other aspects of the work and suggest possible explanations for some of the anomalous results.

METHODS

The microbiological assay of the "*Lactobacillus lactis* Dorner factor" by a tube assay method was first described by Shorb[1], who related the response of the organism to the hæmatopoietic activities of liver extracts in U.S.P. units. Using the same organism, Foster, Lally and Woodruff[2] described a plate assay method.

Many types of microbiological assay have since been evolved. Skeggs, Nepple, Valentik, Huff and Wright[3] used a tube assay method with *L. leichmannii* 4797, and Emery, Lees and Toothill[4] described a similar method and presented a statistical analysis of a (3,3) assay design. Bessel, Harrison and Lees[5] described a cup-plate assay and Burkholder[6] a tube assay, both using the *B. coli* mutant described by Davis. In a later paper Harrison, Lees and Wood[7] described a modified plate assay medium, and reported results obtained with large plates (*cf.* Brownlee, Loraine and Stevens[8]) as well as with the more usual six-cup petri dish. Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin and Jukes[9] described a method using *Euglena gracilis* and Ford[10] introduced the use of the protozoan *Ochromonas malhamensis.*

The work described in this paper has been confined to the use of *B. coli* N.C.T.C. 8134 in plate assays and *L. leichmannii* N.C.T.C. 7854 in both plate and tube assays.

B. coli *Plate Assays*

Harrison and others[7] recommended a (2,2) assay design since, for pure vitamin B_{12}, the log dose/response regression was rectilinear over the dose range 0·005 μg./ml. to 5·0 μg./ml. of vitamin B_{12}. Using five plate replicates they obtained standard errors of 0·14 to 0·23 mm. per zone. They found that the assay was valid for vitamins B_{12a}, B_{12b} and B_{12c} when

the solutions were treated with potassium cyanide, thus converting the compounds into cyanocobalamin before assay.

We have used the *B. coli* plate assay medium of Harrison and others[7] but prefer to use shredded agar which is autoclaved, filtered and added while still hot to the rest of the medium. The complete medium, except for glucose, is distributed in 125-ml. amounts in 150-ml. screw-capped bottles and autoclaved for 30 minutes at 10 lb. pressure. The autoclaved medium can be stored at room temperature and gives satisfactory assays for periods of storage of two months.

An 18-hour inoculum is prepared from a weekly slope culture and grown in 10 ml. of peptone broth. The inoculum density is read on an E.E.L. Colorimeter* and the volume of inoculum to be added is calculated from a chart showing the optimal amount of inoculum that must be used for a given inoculum density to produce the best growth zones.

The bottles of medium are steamed for 30 minutes immediately before use and the clear solution is decanted into a hot conical flask from the insoluble residue of phosphate. To the decanted medium is added 1 ml. of 20 per cent. sterile glucose solution, the flasks and contents are cooled in a water bath to 45–48° C., and the inoculum is then added and thoroughly mixed.

Nine plates are filled on levelling tables using a fast-running bulb pipette graduated at 12·5 ml., allowed to set and then refrigerated for two hours before cutting. Except that plates, pipettes and dilution tubes are dry-sterilised before use, aseptic precautions are unnecessary with this method of assay. Six holes to the plate are cut using a guide-frame to ensure vertical, evenly spaced holes. Careful attention to the cutter has been found to be necessary; silver-steel is preferable to stainless-steel and the cutters are ground and trued on a lathe at weekly intervals. The discs of agar are removed by suction and the plates are refrigerated for about three hours, water being removed from each set of three plates by suction immediately before filling. The holes in the plates are then filled with the appropriate test and standard solutions, the three plates in a set being filled with the same solutions. After incubation at 37° C. for 16 hours the diameters of the zones of exhibition are measured with Lee-Guiness spring-bow dividers to the nearest 0·1 mm.

L. leichmannii *Plate Assay*

The medium used is given in Table I, 230-ml. amounts being stored in screw-capped bottles. The storage life of this medium at room temperature is less than that of the *B. coli* medium and it is renewed monthly.

To each 230-ml. portion of medium cooled to 42–44° C. is added 10 ml. of a saline-washed 18-hour culture of *L. leichmannii* giving an E.E.L. reading at 37 to 40. Except that 25 ml. of medium is added to each plate using a sterilised measuring cylinder the technique of plate preparation is exactly as described for the *B. coli* plate assay.

Usually we carry out *B. coli* and *L. leichmannii* plate assays in parallel

* Evans Electroselenium Ltd., Harlow, Essex.

TABLE I

COMPOSITION OF MEDIUM USED IN *L. leichmannii* PLATE ASSAYS

Component	Amount
Glucose	20 g.
Sodium acetate (hydrated)	20 g.
Sodium citrate	20 g.
Acid hydrolysed casein (A and H)	10 g.
*Salts A	60 ml.
*Salts B	40 ml.
Polysorbate 80	20 ml. of a 10 per cent. solution in neutralised absolute ethanol
L-Cystine	20 ml. of a solution containing 20 μg./ml.
DL-Asparagine	10 ml. " " " 20 μg./ml.
DL-Tryptophane	10 ml. " " " 20 μg./ml.
Xanthine	20 ml. " " " 1 μg./ml.
Adenine	10 ml. " " " 1 μg./ml.
Guanine	10 ml. " " " 1 μg./ml.
Uracil	10 ml. " " " 1 μg./ml.
p-Aminobenzoic acid	5 ml. " " " 1 μg./ml.
Nicotinic acid	2 ml. " " " 1 μg./ml.
Riboflavine	8 ml. " " " 200 μg./ml.
Pyridoxine hydrochloride	0·4 ml. " " " 1 μg./ml.
Calcium *d*-pantothenate	0·4 ml. " " " 1 μg./ml.
Aneurine chloride hydrochloride	0·4 ml. " " " 1 μg./ml.
Biotin	4 ml. " " " 1 μg./ml.
Folic acid	4 ml. " " " 1 μg./ml.
Thioglycolic acid	2 ml.
Sodium chloride	40 g.
Agar	35 g.
Water up to	2000 ml.

* *Salts A*	25 g. K_2HPO_4 25 g. KH_2PO_4	250 ml. of solution.
* *Salts B*	35 g. $MgSO_4.7H_2O$ 7·5 g. $MnSO_4.7H_2O$ 2·1 g. $FeSO_4.4H_2O$	250 ml. of solution.

and it is convenient for each operator to prepare, fill and read three sets of plates with each organism.

The design of the assay, which is similar to that of the *B. coli* plate assay, will be discussed later.

L. leichmannii *Tube Assays*

The *L. leichmannii* tube assay of vitamin B_{12} has been very widely studied. Of particular interest are first the work of Loy, Haggerty and Kline[11,12], who dealt with the effect of reducing agents and with other aspects of the assay, and secondly, the long series of collaborative studies undertaken by the Association of Official Agricultural Chemists[13–15]. Using the medium of the U.S.P. XIV 4th Supplement, and the turbidimetric method of measuring growth the collaborators found on the whole, good inter- and intra-laboratory agreement and the method has now been made official.

Although we have now selected for use the U.S.P. medium (Medium 1) we prefer to measure the bacterial growth by titration of the lactic acid produced rather than by the turbidimetric method.

We have employed one variant of the U.S.P. medium (Medium 2) in which the ascorbic acid has been replaced by thiomalic acid at a concentration of 0·5 g. per litre of medium (double strength), and another variant (Medium 3) in which 6 mg. of potassium cyanide per litre of medium (double strength) has been added to the first variant. This medium is adjusted to pH 6·8 before sterilization, and the others to pH 6·0. All assays are run in triplicate at each dose level.

To each tube is added 5 ml. of medium (double strength) followed by the standard or test solution, and the volume is then adjusted to 10 ml. by the addition of distilled water. The tubes are then autoclaved for 10 minutes only at 10 lb. pressure, cooled, and inoculated with one drop of a dilute suspension of *L. leichmannii* that has been washed 5 times with sterile saline and then suspended in sterile saline to give a reading on the E.E.L. Colorimeter of about 15. The tubes are incubated for 65 hours at 37° C. and the growth is measured by titrating the acid produced to the initial pH value with 0·1N sodium hydroxide, using either bromothymol-blue indicator for Media 1 and 2, or bromocresol-purple for Medium 3.

Results

The results obtained graphically on 14 samples of liver extracts at various stages of production when assayed by five different methods, are given in Table II. Where the assay was valid the results obtained statistically are also given. Where the assay was invalid the reasons for the invalidity are recorded. To illustrate the way in which the results were calculated the values obtained in the *L. leichmannii* plate assay of Sample No. 11, together with the analysis of variance and estimation of potency and fiducial limits, are given in Table III.

TABLE II

COMPARISON OF VITAMIN B_{12} CONTENTS (μg./ml.) OF CRUDE LIVER EXTRACTS OBTAINED BY FIVE METHODS OF ASSAY

Sample	*B. coli* plate assay			*L. leichmannii* plate assay			*L. leichmannii* tube assay								
							Medium 1			Medium 2			Medium 3		
	Graph	Calc.	Valid	Graph	Calc.	Valid	Graph	Calc.	Valid	Graph	Calc.	Valid	Graph	Calc.	Valid
1	0·20	0·20	+	0·33	0·32	+	0·23	0·19	+	0·16	0·17	+	0·21	—	C.C.
2	0·30	0·30	+	0·48	0·50	+	0·24	0·23	+	0·23	0·28	+	0·14	—	P. & C.C.
3	2·2	2·2	+	3·0	3·0	+	1·4	—	O.C.	1·8	1·7	+	1·7	1·7	+
4	1·3	1·3	+	3·6	4·0	+	2·0	2·0	+	1·8	1·6	+	2·1	1·9	+
5	1·6	1·4	+	2·3	—	P. & O.C.	1·3	1·4	+	1·5	1·8	+	2·1	—	C.C.
6	2·6	2·8	+	completely invalid			2·6	2·7	+	3·4	3·4	+	2·4	2·2	+
7	2·4	2·3	+	3·6	3·6	+	3·1	3·1	+	2·8	2·6	+	2·9	2·9	+
8	2·1	2·0	+	4·2	4·4	+	1·7	1·8	+	1·2	—	O.C.	1·8	—	C.C.
9	1·8	1·8	+	3·3	3·6	+	1·7	1·7	+	1·8	1·7	+	3·4	3·4	+
10	1·8	1·9	+	3·0	3·2	+	1·8	1·8	+	2·3	—	O.C.	2·9	2·8	+
11	2·2	1·9	+	2·7	2·6	+	1·7	1·8	+	1·2	—	O.C. & P.	1·2	—	C.C.
12	4·0	4·0	+	7·1	6·8	+	3·4	3·6	+	3·6	3·7	+	2·9	—	C.C.
13	4·1	4·0	+	7·0	6·5	+	3·5	3·5	+	4·5	—	O.C. & P.	5·2	—	C.C.
14	2·9	2·6	+	4·2	4·1	+	2·9	2·8	+	3·3	3·1	+	1·3	1·5	+

Graph: Result estimated graphically, fitting regression lines by eye.
Calc.: Calculated result where assay is valid.
Valid: State of validity. If invalid: P = lack of parallelism; O.C. = opposed curvature; C.C. = combined curvature.

TABLE III

POTENCY OF SAMPLE NO. 11 (see Table II) BY THE *L. leichmannii* PLATE ASSAY

	Diameters of zones in 0·1 mm.						Coefficients of orthogonal contrast				
	Standard (1 μg./ml.) diluted			Test solution (1/3 dilution) diluted							
	1/20	1/10	1/5	1/20	1/10	1/5	Lp	L_1	L_1'	L_2	L_2'
Zone Size	113 111 115	130 130 129	149 149 140	111 108 111	128 124 127	143 141 142	−10 −17 −4	68 71 56	4 5 −6	0 1 −4	4 −1 −2
Totals	339	389	438	330	379	426	−31	195	3	−3	1

Analysis of Variance

Adjustment for mean = 294144·5

Source of variation	S of S	d.f.	M.S.	F	P
Preparations	53·4	1	53·4	7·98	<0·05
Regression	3168·8	1	—	—	—
Parallelism	0·8	1	0·8	<1	—
Combined curvature	0·3	1	0·3	<1	—
Opposed curvature	0·0 (3)	1	0·0 (3)	<1	—
Between doses	3223·3	5	—	—	—
Between plates	12·3	2	6·15	<1	—
Error	66·9	10	6·69	—	—
Total	3302·5	17	—	—	—

In the analysis of variance the subdivision of the between doses sum of squares has been carried out by the method of detached coefficients or orthogonal contrast (Bliss and Marks[14]) Lp, L_1, L_1', L_2, L_2', have their usual significance and refer, in turn, to the five contrasts in the order shown in the above Table.

Estimation of Potency and Validity.

The contrast for preparations is significant at P = 0·05 but not at P = 0·01. None of the contrasts for parallelism, combined curvature or opposed curvature is significant, however, and the assay may be considered valid.

Using the accepted notation (see Finney[13], Chapter V).

Mean potency (R) = antilog $\frac{d(k^2 - 1)\,Lp}{6L_1}$

In all assays discussed in this paper the log dose interval (d) = 0·3010 and k (number of doses of standard and test material) = 3.

Then $\log R = \frac{4d\,Lp}{3L_1}$ and R = 0·86.

Since the test solution was initially diluted 3-fold and the standard contained 1 μg./ml. of cyanocobalamin, then:—

Potency of extract = 3 × 0·86 = 2·58 μg./ml.

The index of significance of the slope (g) = $\frac{N t^2 s^2 (k^2 - 1)}{12 L_1^2} = 0{\cdot}0104$.

Since g is small the 95 per cent. fiducial limits are given by:—

$$\text{Log RL, RU} = \left[\frac{d(k^2-1)}{6L_1}\left\{Lp \pm t\sqrt{Ns^2\left[1 + \frac{(k^2-1)Lp^2}{12L_1^2}\right]}\right\}\right]$$

$$= \frac{4d}{3L_1}\left\{Lp \pm t\sqrt{18 s^2 (1 + 2Lp^2/3L_1^2)}\right.$$

From the error mean square (analysis of variance) s^2 = 6·69 and for 10 degrees of freedom t = 2·228 at P = 0·05.

Whence 95 per cent. fiducial limits are 2·31 — 2·91 μg./ml. (89·5–112·8 per cent.).

Table IV gives an analysis of the results obtained with Sample No. 11 by the *B. coli* plate assay, the calculations being carried out in a similar manner to those in Table III.

For the three *L. leichmannii* tube assays the analysis was carried out in a different way. The value of s^2 was obtained in the normal way

TABLE IV

POTENCY AND FIDUCIAL LIMITS OF SAMPLE NO. 11 (see Table II) BY THE *B. coli* PLATE ASSAY

The results given below were analysed by the method described for the *L. leichmannii* plate assay (see also under "Discussion").

Contrasts and mean squares from analysis of variance

Contrast	Sum	Mean square	F	P
Preparations	−91	460·055	586·80	<0·01
Regression	185	2852·083	—	—
Parallelism	5	2·083	2·66	>0·1
Combined curvature ..	−11	3·361	4·29	⩾0·05
Opposed curvature ..	1	0·028	<1	—
Error		0·784		

The sums of the contrasts are the values of Lp, L_1, etc., used in the determinations of potency and fiducial limits.
Potency = 1·91 μg./ml.
95 per cent. fiducial limits 1·82–2·00 μg./ml. (95·3–104·7 per cent.).

TABLE V

POTENCY AND FIDUCIAL LIMITS OF SAMPLE NO. 11 (see Table II) BY THE *L. leichmannii* TUBE ASSAY

In all tube assays a standard solution containing 0·02 mμg./ml. of cyanocobalamin was employed and the test material was diluted accordingly (see text).

U.S.P. Assay (*Medium* 1)—

Contrasts and Standard Errors

Contrast	Sum	Standard error	Ratio: sum/standard error
Preparations	44	3·18	13·84
Regression	106	2·58	41·09
Parallelism	−2	2·58	0·78
Combined curvature ..	8	4·47	1·79
Opposed curvature ..	4	4·47	1·79

s^2 = 0·556 (from within dose groups sum of squares). For 12 degrees of freedom t = 2·18 and none of the contrasts for parallelism, combined curvature or opposed curvature is significant. Then potency = 1·76 μg./ml.
95 per cent. fiducial limits = 1·66 – 1·87 μg./ml. (94·3–106·3 per cent.).

Medium 2—

Contrasts and Standard Errors

Contrast	Sum	Standard error	Ratio: sum/standard error
Preparations	−96	4·38	21·92
Regression	195	3·55	54·93
Parallelism	9	3·55	2·54
Combined curvature ..	7	6·16	1·14
Opposed curvature ..	21	6·16	3·41

s^2 = 1·06 (from within dose groups sum of squares). t = 2·18 and the contrasts for parallelism and opposed curvature are significantly high. This assay was therefore invalid and no further calculations were carried out.

Medium 3—

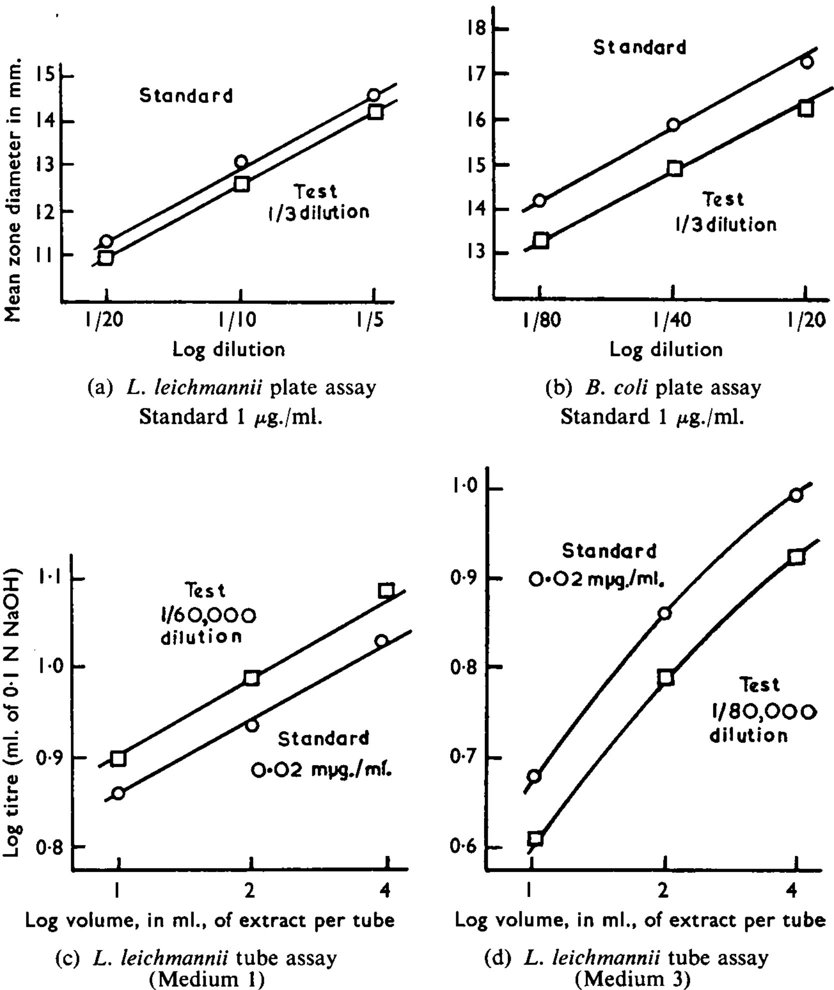
Contrasts and Standard Errors

Contrast	Sum	Standard error	Ratio: sum/standard error
Preparations	−65	4·14	15·70
Regression	187	3·36	55·65
Parallelism	−3	3·36	0·89
Combined curvature ..	−45	5·83	7·72
Opposed curvature ..	−7	5·83	1·20

s^2 = 0·944 (from within dose groups sum of squares).
Potency = 1·15 μg./ml.
Fiducial limits not estimated (see Discussion).

from within dose groups and the significance of the five contrasts was then estimated by a "t" test by dividing each contrast by its standard error and comparing with $t = 2{\cdot}18$, as in this instance there are 12 degrees of freedom for the error term. The procedure is described elsewhere in detail[16,17]. We now use the same analysis of variance employed for the plate assay except that there is of course no term for difference between plates. The same formula as that used for the plate assays is used for the estimation of fiducial intervals. The results are given in Table V.

Figure 1 gives the graphical plots of the four valid assays of Sample No. 11. The plot of the thiomalic acid assay is not given, as this assay was invalid on two counts.



(a) *L. leichmannii* plate assay
Standard 1 μg./ml.

(b) *B. coli* plate assay
Standard 1 μg./ml.

(c) *L. leichmannii* tube assay
(Medium 1)

(d) *L. leichmannii* tube assay
(Medium 3)

FIG. 1. Graphical representation of results obtained in the assay of Sample No. 11 (See Table II) by four different methods.

Discussion

For the last nine years many workers have used cup-plate and tube assay methods for estimating vitamin B_{12}, with either *B. coli*, *L. lactis* Dorner or a strain of *L. leichmannii* as the test organism. The methods, developed originally for the assay of the pure vitamin, have been applied, often without modification, to the assay of natural products with the pure vitamin as standard. In particular the assay of liver extracts by this means has been widely practised and the anomalies encountered with this type of material have been mentioned in several papers[18–21].

Dawbarn and Hine[20] used a four point (2,2) plate assay at levels of 0·005 μg./ml. and 0·2 μg./ml. of vitamin B_{12} with *B. coli* as test organism as well as a tube assay method with *L. leichmannii*. They reported that sheep liver extracts almost invariably gave invalid assays shown by a lack of parallelism between the dose-response curves of the standard and test solutions, the latter tending to give a significantly lower slope. One assay is reported on rumen contents where they interpolated three doses between their usual 40-fold dilutions and found a curvilinear regression with a significantly higher slope through the three greatest concentrations. For liver extracts, agreement at the "1/5 level" between *B. coli* plate assays and *L. leichmannii* tube assays was reasonably satisfactory, the extracts being prepared by macerating 10 g. of liver, extracting with water and diluting to 250 ml.

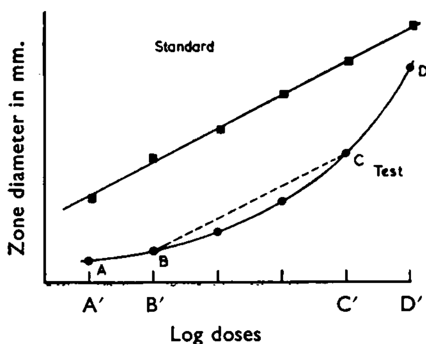


Fig. 2. Hypothetical curvilinear log dose response regression given by test material, showing apparent validity at B—C.

We consider these observations to be of considerable practical importance. A limitation of the (2,2) design is that it cannot show departure from linearity. Figure 2 illustrates a hypothetical case where a rectilinear dose-response for the standard is compared with a curvilinear dose-response for the test material. If it so happened that samples were usually diluted within the dose-range B′–C′ an apparently valid series of assays could be obtained and the occasional change in slope and hence non-parallelism such as arises in the range A–B, C–D, could be explained as random variation.

Figure 3 shows a pair of dose-response curves that have been obtained with a liver extract assayed over a wide range of dilutions by the *B. coli* plate method. A (2,2) design employing any pair of alternate points at a concentration of test material greater than 0·05 μg./ml. would have given an apparently linear non-parallel response (e.g. points A and B, as joined on the graph). This type of graph has been obtained for many extracts and thus, whereas for simple solutions the (2,2) design may be

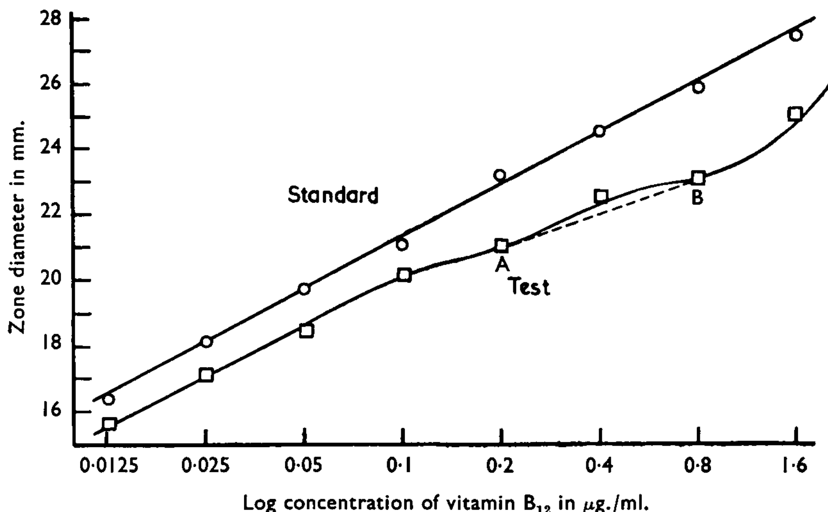


FIG. 3. Graphical representation of results obtained in the assay of a liver extract by the *B. coli* plate method over a wide range of vitamin B_{12} concentrations, showing curvilinear regression of test material and rectilinear regression[12] of standard (1 μg./ml.).

satisfactory provided a careful check is kept on parallelism (see "Antibiotics: Properties and Uses"[22], p. 82), the design is basically unsound for complex mixtures where the test dose-response regression may not be rectilinear over the whole range in which the standard gives a linear response.

Jerne and Wood[23] quote three assumptions that must be made if a body of data is to be regarded as yielding a fundamentally valid assay:

(1) The hypothesis of the validity of the experimental design.

(2) The hypothesis of the existence of a single-valued dose-response relationship.

(3) The hypothesis of similarity of the test and standard preparations. As has been shown, condition (3) cannot be assumed to hold for liver extracts, and similar considerations apply to assays of other complex materials for various ingredients. It would seem prudent to test any new preparation over a wide range of concentrations against a standard before assuming that a simple assay design is adequate.

Reference to Figure 3 shows that within a range of concentrations from 0·0125 μg./ml. to 0·05 μg./ml. of vitamin B_{12}, the standard and test materials gave parallel dose-response curves for the *B. coli* plate assay of liver extracts. Applying exactly the same considerations to the *L. leichmannii* plate assay the effective range was found to be 0·05 to 0·2 μg./ml. of vitamin B_{12}.

As a result of these findings a (3,3) assay design has been adopted. Thus for *B. coli* plate assays 3 doses of the standard at 0·0125, 0·025 and 0·05 μg./ml. of vitamin B_{12} are employed and 3 doses of the test solution at equivalent concentrations. For the *L. leichmannii* plate assays

the concentrations of vitamin B_{12} used are 0·05, 0·10 and 0·20 μg./ml. Fiducial limits (95 per cent.) of about $\pm$ 10 per cent. are usually obtained.

All assays are computed statistically and examined for departure from parallelism and combined and opposed quadratic curvature as described under "Results". As Table II shows, there is generally good agreement between the graphical and calculated results except where one of the contrasts L_1', L_2 or L_2' approaches significance. For example, in the *B. coli* plate assay (Table IV) the contrast for combined curvature is borderline, and in this instance the agreement between the two results is not so good as in the *L. leichmannii* plate assay.

Using *L. leichmannii* for tube assays very good results have been obtained with the U.S.P. medium over the range 0·02 to 0·10 mμg. of vitamin B_{12} per tube, only one out of the 14 samples included in Table II giving an invalid result; the assay could not be repeated in this instance owing to loss of material. A (3,3) design has been adopted with standard solutions containing 0·02, 0·04 and 0·08 mμg. of vitamin B_{12} per tube and corresponding dilutions of the test solution. The results generally agreed with those obtained in the *B. coli* plate assay.

Figure 1c and the corresponding analysis in Table V show the results obtained in the assay of Sample No. 11.

Medium 2 has given rather more trouble. Preliminary investigations suggested that the same range of dilutions should be used as for the U.S.P. medium. Table II shows, however, that invalid assays were quite often obtained with this medium, four out of the last seven assays being invalid. Where the assays were valid the results agreed fairly well with those obtained by the *B. coli* plate and U.S.P. tube assay methods. Invalidity showed as opposed curvature and as lack of parallelism, an observation recalling the work of Loy and others[11,12] who gave a graph of the results obtained with the U.S.P. medium to which thiomalic acid had been added; this graph would certainly not have been rectilinear had it been plotted on a log-log scale.

Medium 3 gave very variable results, although other workers have claimed that cyanide improves the validity and gives higher results. We have found that the response obtained was sometimes higher and sometimes lower, and that in many instances it was not possible to obtain a rectilinear dose-response curve for either test or standard solution. Figure 1d and the corresponding analysis in Table V for Sample No. 11 show a typical response of this type. A (2,2) assay for this material would have given an apparently valid result and in fact would give a good approximation of the potency. Even where the samples did give valid assays, the results varied widely from stage to stage of the extraction process and no attempt has been made to equate the mean quadratic regression, as checking pairs of quadratic regressions for validity is tedious.

By treating this type of assay as though it were valid and by using the same method of analysis as that applied to the linear regression, it can be shown that the contrast for opposed curvature is always without significance and that "lack of parallelism" is also nearly always absent. The latter point can be partially confirmed by treating the assay as two

(2,2) designs taking the three points in pairs and showing that the regressions so obtained are almost invariably parallel. It is admitted that this treatment is far from rigorous but it was not thought worthwhile to investigate the assay further. (For further discussion on this problem see Finney, "Statistical Method in Biological Assay"[17], p. 107 *et seq.*)

As with the plate assay methods, the potencies obtained by the tube assays are estimated graphically. This method checks any gross errors in computation and avoids the waste of time involved in working through the calculation of an obviously invalid assay. In the case of Sample No. 11 (Table II) for example, the computation of the contrasts and their standard errors would not normally have been carried out on the results obtained with Medium 2 as a graphical plot showed that the assay was clearly invalid.

We consider that the U.S.P. tube assay is the most reliable method of estimating the vitamin B_{12} content of liver extracts and gives results that are easily interpreted. It is not proposed to discuss in this paper the complications introduced by the use of different reducing agents in the medium. (See Loy and others[11,12], Denton and Kellog[24], Shenoy and Ramasarma[19], Dawbarn and Hine[20].) In view of our findings, however, that the *L. leichmannii* tube assay gives results in close agreement with the *B. coli* plate assay we believe that the latter may give results in closer agreement with the clinical activity than has generally been conceded.

The *L. leichmannii* plate assay presents a problem in that apparently valid assays on all counts were obtained on twelve out of the fourteen samples of Table II, and yet the results were invariably higher than those obtained by the *B. coli* plate assay, occasionally being twice and in one instance three times as high.

Use of Internal Standard

An obvious possibility, when faced with the problem of assaying a complex product, is to use as standard a preparation that closely resembles the preparation being assayed. Sometimes as in penicillin assays, it is recommended that the active ingredient be extracted from the mixture containing it and assayed against the pure standard. Alternatively, interfering substances can often be removed by solvent extraction, enzymatic action, adsorption or other means. Occasionally, however, neither of these methods is satisfactory because the active ingredient appears to exist in the form of a complex with some other constituent of the preparation, the complex then giving a response different from that given by the substance in the pure state. Such a complex of vitamin B_{12} may be present in liver extracts[21,25–27].

An internal standard has sometimes been used to overcome the difficulties introduced by the presence of interfering substances. Thus in the microbiological assay of aneurine by the method of Fitzgerald and Hughes[28], the vitamin is destroyed in an aliquot portion of the test solution by autoclaving with sodium sulphite, excess sulphite being removed by titration with hydrogen peroxide. Successive amounts of aneurine are

then added to portions of the aneurine-free preparation, giving a series of internal standards against which the untreated preparation is assayed.

Beck[29] described a method of this type for the assay of liver extracts in which treatment with acid was used to hydrolyse the vitamin B_{12}, thus rendering it microbiologically inactive. We investigated the use of internal standards obtained by hydrolysing liver extracts with sulphuric acid, sodium carbonate or sodium hydroxide followed by the addition of pure cyanocobalamin. Beck's original method, which was developed in conjunction with an *L. leichmannii* tube assay, was found to be very cumbersome, requiring a great deal of troublesome preparative work and the use of aseptic precautions throughout. Modified to suit the plate assay method, however, it has given encouraging results with both *B. coli* and *L. leichmannii* as test organisms.

Alkali-stable Factors

It is possible that the presence of alkali-stable factors in liver extracts may be partly responsible for some of the invalid results obtained[18,24]. Further discussion of this problem is reserved for a later paper.

Summary

1. Experimental methods are given for the assay of cobalamins in liver extracts using plate and tube assay methods with *B. coli* N.C.T.C. 8134 and *L. leichmannii* N.C.T.C. 7854 as test-organisms.

2. The use of a (2,2) design for complex materials is criticised and the adoption of a (3,3) design is advocated for liver extracts and similar materials.

3. Details of the experimental design and statistical analysis employed are related to the assay of fourteen samples of liver extract and the results are discussed.

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