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

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THE first indication that vitamin B_{12} was a growth factor for micro-organisms was provided by the work of Shorb,[1] who showed that it was essential for the growth of *Lactobacillus lactis* Dorner and that the amount of growth was proportional to the amount of vitamin B_{12} present. Shorb developed a turbidimetric method of assay, but this has since been found to give somewhat unsatisfactory results; in the first place the organism is rather unstable,[2] secondly, it will apparently grow without vitamin B_{12} under anaerobic or reducing conditions,[3,4] and, thirdly, thymidine and other deoxyribosides also stimulate the growth of the organism, making it essential to separate these from vitamin B_{12} prior to carrying out the assay.

Cuthbertson, Pegler and Lloyd[5] obtained more satisfactory results with *L. lactis* Dorner by using the cup-plate method of Bacharach and Cuthbertson.[6] In this method petri dishes containing a solid medium seeded with the test organism are used, holes are cut into the agar and filled with dilutions of the solution to be tested and the plates are then incubated. The organism forms zones of stimulation around the holes containing the vitamin B_{12} solution and the diameters of these zones are proportional to the concentration of the vitamin. Using this method Cuthbertson *et al.*[5] obtained satisfactory results with fiducial limits of ± 15 per cent. ($P = 0{\cdot}05$)—sufficiently narrow for routine purposes. Thymidine and other deoxyribosides when present form zones of stimulation somewhat fainter and more diffuse than those formed by vitamin B_{12} and these may interfere with the accurate measurement of the latter. Interference due to deoxyribosides can be eliminated by paper chromatography as described by Winsten and Eigen[7] and by Smith and Cuthbertson.[8]

Vitamin B_{12} is also necessary for the growth of *Lactobacillus leichmannii*; on the whole this has given more satisfactory results than has *L. lactis* in the assay of the vitamin turbidimetrically.[9,10,11] Like *L. lactis*, however, *L. leichmannii* is also stimulated by thymidine and other deoxyribosides.

We have used *L. leichmannii* for the assay of vitamin B_{12} by the cup-plate method and obtained satisfactory results with relatively pure solutions of the vitamin. With certain crude liver extracts, however, difficulties were encountered that have apparently not been previously reported.

In the turbidimetric assay of vitamin B_{12} by means of *L. leichmannii*, Hoffmann *et al.*[10] recommend that a second assay of the vitamin B_{12} solution should be carried out after digestion with alkali, e.g., by boiling the solution for 30 minutes after adjustment to *p*H 10. The difference

between the value thus obtained and that obtained on the untreated solution is said to be proportional to the amount of vitamin B_{12} present, since this is completely destroyed by alkali under the conditions used whereas thymidine and other deoxyribosides are unaffected.

In assaying crude liver extracts by the cup-plate method 3, or even 4, zones could sometimes be seen. The largest of these was easily distinguished from the others by reason of the less dense growth and was identical in appearance with the zone produced by thymidine, a specimen of which was kindly supplied to us by Professor A. R. Todd. This zone may occasionally interfere with the measurement of the other zones, but normally the cup-plate method enables vitamin B_{12} to be assayed in presence of thymidine without difficulty. In order to determine which of the other zones was due to vitamin B_{12} the solution was re-tested after digestion with alkali; it was found that the innermost zone generally disappeared and this zone was therefore assumed to be due to vitamin B_{12}. The results were accordingly calculated from the diameter of this zone. Sometimes, however, there appeared to be little difference in the appearance of the plates before and after alkaline hydrolysis, whilst occasionally one of the intermediate zones disappeared, and in such instances the results calculated from the diameter of the innermost zone were unsatisfactory for, when known amounts of vitamin B_{12} were added to the test solution and the assay repeated, the results obtained did not agree with the amount of vitamin B_{12} known to be present.

Winsten and Eigen[7] showed that vitamin B_{12} could be separated from thymidine and other deoxyribosides by paper chromatography. When the developed paper chromatogram was applied to agar plates seeded with *L. leichmannii* at least 6 zones of stimulation were observed; a similar result was obtained by Cuthbertson and Smith[8] with *L. lactis*. We applied this method to crude liver extracts and obtained several zones of stimulation. We had no difficulty in identifying the zones due to vitamin B_{12} and vitamin B_{12a}. However when the extracts were digested with alkali and the hydrolysates chromatographed on filter paper strips we were surprised to find that the chromatograms were apparently unchanged, a zone of stimulation being still present at the site normally occupied by the different forms of vitamin B_{12}. This indicated that in the crude liver extracts under examination there was present an alkali-stable growth factor for *L. leichmannii* with an R_F value close to that of vitamin B_{12} itself. In the turbidimetric assay of such crude extracts the method of Hoffmann *et al.* for correcting for the presence of alkali-stable substances would presumably give satisfactory results, but merely removing the deoxyribosides by paper chromatography would still give too high a result for the vitamin B_{12} content.

Attempts have been made to separate the alkali-stable factor from vitamin B_{12} in order to obtain more consistent assay results. Crude liver extracts were applied to columns of various ion exchange resins and some evidence of separation was obtained with Zeokarb 215. Better separation was obtained, however, by extraction with phenol which removed the vitamin B_{12}, leaving most of the alkali-stable factor in the aqueous phase.

The most satisfactory method proved to be that of Peeler and Norris,[12] in which the extract acidified to *p*H 2 was extracted repeatedly with a mixture of phenol and butanol equilibrated with water. Using this method the phenol-butanol extract gave a zone of stimulation that completely disappeared on alkaline hydrolysis, whereas the zone of stimulation given by the aqueous phase was unaffected by treatment with alkali.

In general it would appear that the results obtained in the microbiological assay of vitamin B_{12} in crude extracts by means of *L. leichmannii* should be interpreted with great caution. One of the great advantages of the cup-plate method over the turbidimetric method is that the former will discriminate between different growth factors (and inhibitors) present, whereas with the latter the amount of growth is a measure of the combined effect of each growth factor and growth inhibitor present. We believe that many of the results reported in the literature for the vitamin B_{12} contents of various natural products and crude extracts may be too high owing to the presence of other growth factors for which no allowance has been made.

It has been reported[13,14] that a mutant of *Escherichia coli* responds to methionine and vitamin B_{12} but not to thymidine. We have investigated the assay of crude liver extracts with this organism using the cup-plate method, and compared the results obtained with those given by *L. leichmannii*. In general the *E. coli* mutant gave results in good agreement with those given by *L. leichmannii*, although with some crude liver extracts, *E. coli* sometimes gave lower results. Such extracts also gave complex zones of stimulation with *E. coli*, although it has been stated that this organism responds only to methionine and to vitamin B_{12}. One of the zones of stimulation appeared to be due to the same alkali-stable factor as that which stimulated the growth of *L. leichmannii*. However, whereas *L. leichmannii* gave fairly satisfactory recoveries when known amounts of vitamin B_{12} were added to a solution of the alkali-stable factor freed from vitamin B_{12}, the results obtained with *E. coli* were much lower than would have been expected from the amount of vitamin B_{12} known to be present. This suggests that the solution contains in addition to the alkali-stable substance that stimulates the growth of both *L. leichmannii* and *E. coli* another substance that interferes with the utilisation of vitamin B_{12} by *E. coli*. This presumably explains the low results sometimes observed in the assay of the cruder types of liver extract by means of the *E. coli* mutant.

It has been suggested that the multiple zones of stimulation observed with the *E. coli* mutant might have been due to the formation of a mixture of variants in our particular culture, but precisely similar results have been obtained with a purified strain kindly sent to us by Dr. W. F. J. Cuthbertson.

Experimental

Assays with L. leichmannii. The medium now used as a routine has the composition given in Table I.

The *p*H of the medium can be varied between 5·0 and 6·5 without

affecting the diameters of the zones of stimulation; normally a *p*H of 5·5 is used. The test solutions are made up in phosphate buffer solution of *p*H 5·2.

Stock cultures of the organism are maintained on a soya bean medium and up to the present no indication has been obtained that the response of the organism to vitamin B_{12} has varied since the work was commenced. Cultures for assay are prepared by transferring a loopful of the stock culture to 10 ml. of the basal medium from which salt and agar have been omitted and to which has been added 2 per cent. of crude liver extract ("Hepolon"). The tube is incubated for 18 hours and then centrifuged for 10 minutes. The supernatant liquid is withdrawn and replaced by 10 ml. of 0·86 per cent. saline. The tube is then well shaken and the turbidity measured in an Eel nephelometer, and the result compared with that of an arbitrary standard. Each culture is diluted if necessary in order to obtain approximately the same density for each test. Too light an inoculum tends to give large zones with sparse growth.

An adequate amount of the basal medium is sterilised, cooled to 50° C. and the inoculum added with stirring. The requisite number of sterile 9 cm. petri dishes are put on to a level surface, and 25 ml. of the medium poured on to each plate, giving a depth of medium that in our hands has given the most satisfactory results. When the agar has set, 6 holes are cut in each plate with a sterile cork borer 6 mm. in diameter, any liquid that has collected in the holes is then withdrawn by suction, and 5 of the holes are then filled with the solutions to be tested, and the sixth hole with a standard solution (0·2 μg./ml.) prepared from crystalline vitamin B_{12} e.g., "Anacobin"). The plates are incubated at 37° C. for 18 hours. With each set of plates at least 2 plates are included containing standard solutions only, at concentrations of 0·03, 0·05, 0·1, 0·2, 0·4 and 0·6 μg./ml.

TABLE I

COMPOSITION OF *L. leichmannii* ASSAY MEDIUM

Component	Amount
Glucose	10 g.
Sodium acetate	10 g.
Sodium citrate	10 g.
Acid-hydrolysed casein	5 g.
Dipotassium hydrogen phosphate	3 g.
Potassium dihydrogen phosphate	3 g.
Magnesium sulphate ($7H_2O$)	2·8 g.
Manganese sulphate	0·6 g.
Ferrous sulphate ($7H_2O$)	0·17 g.
Tween 80	1·0 g.
Sodium chloride	·20 g.
L-Cystine	0·2 g.
Asparagine	0·1 g.
DL-Tryptophane	0·1 g.
Thioglycollic acid	1 ml.
Xanthine	10 mg.
Adenine	10 mg.
Guanine	10 mg.
Uracil	10 mg.
p-Aminobenzoic acid	2·5 mg.
Nicotinic acid	1·0 mg.
Riboflavine	0·8 mg.
Pyridoxine	0·2 mg.
Calcium pantothenate	0·2 mg.
Aneurine	0·2 mg.
Biotin	0·002 mg.
Pteroylglutamic acid	0·002 mg.
Agar	18 g.
Distilled water	1·0 l.

The diameters of the zones of stimulation obtained with these standard solutions are plotted against the logarithms of the concentrations of vitamin B_{12} and this gives the day's standard curve, from which the vitamin B_{12} contents of the unknown solutions are calculated. The inclusion of a standard solution on each plate enables any variation from plate to plate to be detected. A typical standard curve is shown in Figure 1A.

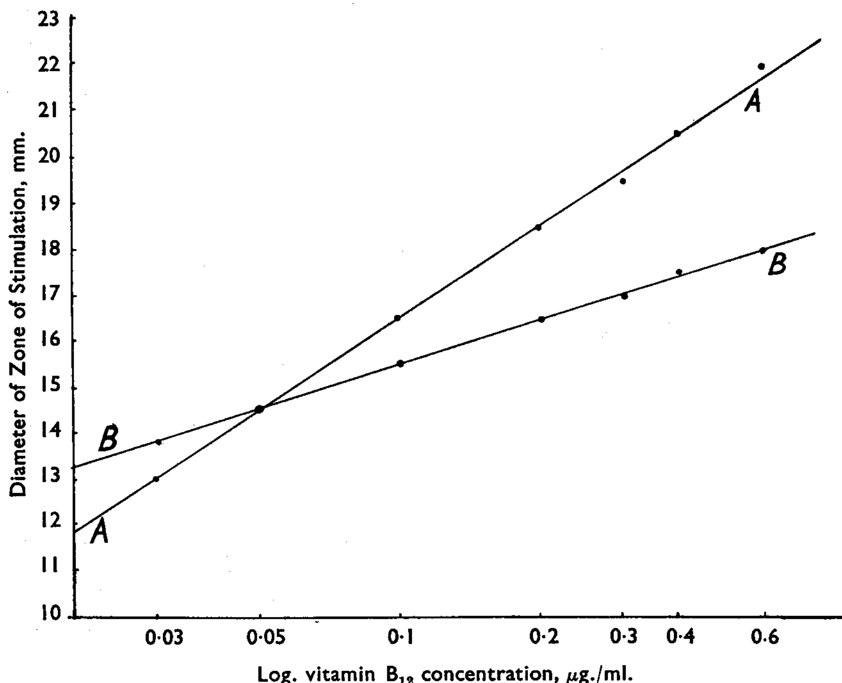

FIG. 1. Standard curves obtained by plotting the diameters of the zones of stimulation against the logarithms of the concentrations of vitamin B_{12} solution using (A) *L. leichmannii* and (B) the *E. coli* mutant, as test organisms.

Assays with E. coli. The medium used was that described by Davis and Mingioli,[13] and the organism was maintained in accordance with the recommendations of Bessell *et al.*[14] The experimental procedure was the same as with *L. leichmannii*, except that the medium was adjusted to *p*H 7·2 and the test solutions were diluted with a phosphate buffer solution of *p*H 7·0 to 7·2. A typical standard curve is shown in Figure 1B.

Results. Assays were carried out on a number of liver extracts made in Great Britain. The results, recorded in Table II, indicate that with most extracts *L. leichmannii* and *E. coli* give results that are in reasonably close agreement. Several of the crude liver extracts, however, gave lower results with *E. coli* and gave more than one zone with both organisms; one or two of the extracts gave at least 3 zones. The results are calculated on the assumption that the innermost zone is due to vitamin B_{12}.

Recovery experiments. Table III illustrates the results obtained when known amounts of vitamin B_{12} were added to liver extracts that gave only

TABLE II

POTENCIES OF LIVER EXTRACTS ASSAYED BY *L. leichmannii* AND *E. coli*

Extract and type	Stated potency	Potency found (μg./ml.)		Remarks
		L. leichmannii	*E. coli*	
A.1 Crude	—	1·7	1·0	Small zone after hydrolysis
A.2 Concentrated	—	4·5	4·3	Faint zone after hydrolysis
C. Crude	2 U.S.P. units	1·5	1·3	Faint zone after hydrolysis
E. Concentrated	12 μg./ml.	10·5	11·5	Faint zone after hydrolysis
H.1 Crude	—	0·5	0·1	Three zones, only one disappeared on hydrolysis
H.2 Concentrated	—	7·0	7·5	Faint zone after hydrolysis
H.3 Crude	2 μg./ml.	0·6	0·6	Faint zone after hydrolysis
L. Crude	—	1·2	0·8	Two zones, one remained after hydrolysis
N. Concentrated	12 μg./ml.	9·0	9·5	Faint zone after hydrolysis
P.1 Crude	1·5 μg./ml.	1·6	1·2	Two zones, haze after hydrolysis
P.2 Concentrated	20 μg./ml.	22·5	24	One zone, disappeared on hydrolysis
P.3 Crude	4 μg./ml.	4·5	4·0	Three zones; large zone left after hydrolysis

TABLE III

RECOVERY OF VITAMIN B_{12} ADDED TO LIVER EXTRACTS

Extract	Vitamin B_{12} content (μg./ml.)			
	L. Leichmannii assays		*E. coli* assays	
	Calculated	Found	Calculated	Found
A.2 + 1 μg. B_{12}	0·38	0·35	0·38	0·36
C. + 1 μg. B_{12}	0·32	0·28	0·31	0·28
E. + 1 μg. B_{12}	0·40	0·44	0·44	0·36
H.2 + 1 μg. B_{12}	0·52	0·44	0·52	0·50
H.3 + 1 μg. B_{12}	0·29	0·33	0·29	0·28
L. + 1 μg. B_{12}	0·40	0·35	0·34	0·28
N. + 1 μg. B_{12}	0·38	0·44	0·38	0·36
P.4 + 2 μg. B_{12}	0·5	0·5	0·8	0·8
P.4 + 6 μg. B_{12}	0·9	1·0	1·2	0·8
P.4 + 10 μg. B_{12}	1·3	1·6	1·6	1·2
P.5 + 1 μg. B_{12}	0·35	0·30	0·30	0·15
P.5 + 2 μg. B_{12}	0·55	0·40	0·50	0·40

2 zones with *L. leichmannii*, one due to vitamin B_{12} and the other to thymidine, and one zone with *E. coli*. The values thus obtained are in fair agreement with the values calculated from the amounts of vitamin B_{12} added, and indicate that the presence of thymidine in such extracts does not seriously interfere with measurement of the vitamin B_{12} zone. Table IV records similar results with an extract that gave a more complex system of zones and shows that unsatisfactory results are obtained whichever of the two inner zones is used as the basis of calculation.

Paper Chromatography. In view of the unsatisfactory results obtained with several of the cruder liver extracts, the method of Winsten and Eigen[7] was investigated. A 10-μl. spot of the test solution was applied to a strip of filter paper which was then dried and developed from the top

TABLE IV

RECOVERY OF VITAMIN B_{12} ADDED TO CRUDE LIVER EXTRACTS

Extract	Result calculated from	Vitamin B_{12} content (µg./ml.)			
		L. leichmannii assays		*E. coli* assays	
		Calculated	Found	Calculated	Found
H.3	Zone 1	—	0·2	—	0·25
	Zone 2	—	2·6	—	5·1
H.3 + 0·1 µg. B_{12}	Zone 1	0·15	0·1	0·2	0·1
	Zone 2	1·35	3·0	2·6	2·2
H.3 + 0·4 µg. B_{12}	Zone 1	0·3	0·15	0·3	0·1
	Zone 2	1·5	3·0	2·7	3·9
H.3 + 1·0 µg. B_{12}	Zone 1	0·6	0·1	0·6	0·2
	Zone 2	1·8	2·1	3·1	0·5

downwards with *n*-butanol saturated with water in the usual way. The paper strip was then dried and transferred to an agar plate seeded with *L. leichmannii.* The paper was either removed after a few minutes and the plate then incubated or the plate was incubated with the paper still in position. The second procedure gave the better defined zones, although both gave substantially the same results. The position of the vitamin B_{12} zone and the amount of vitamin B_{12} were determined by comparison with the chromatogram obtained when a 10-µl. spot of a standard solution of vitamin B_{12} was treated in the same way. Generally this standard solution gave a single zone, but occasionally a smaller second zone appeared slightly below it; this is presumably due to the formation of vitamin B_{12a} during chromatography.[15] Both these zones were absent after alkaline hydrolysis, as was the top zone of the chromatograms of all the concentrated liver extracts examined. With some crude liver extracts, however, the appearance of the chromatogram after alkaline hydrolysis was almost the same as before hydrolysis. In Table V are recorded some of the results obtained with different liver extracts when assayed directly and after paper chromatography. There is not very good agreement between the two sets of results, and it is by no means certain which method gives the more reliable estimate.

TABLE V

COMPARISON OF VITAMIN B_{12} CONTENT OF LIVER EXTRACTS, AS ESTIMATED BY DIRECT ASSAY AND BY ASSAY AFTER CHROMATOGRAPHY

Extract and type		Vitamin B_{12} content (µg./ml.)	
		Direct assay	Assay after chromatography
H.4	Crude	0·4	1·4
H.5	Crude	0·9	1·5
H.6	Concentrated	3·4	2·8
H.7	Concentrated	5·3	4·3
H.8	Crude	0·9	1·5
H.9	Crude	1·6	2·8
H.10	Crude	1·0	2·2
H.11	Crude	1·0	2·2
H.12	Crude	0·5	0·7
W.	Concentrated	4·0	5·0, 6·0
A.3	Concentrated	1·7, 2·0	1·2

Effect of Cyanide, Reducing Agents, etc. When vitamin B_{12} is assayed turbidimetrically with *L. leichmannii* the depth of the medium in the assay tubes affects the response,[11,16] because the vitamin B_{12} requirement of the organism depends on the E_H of the medium. Hoffmann *et al.*[10] reported that thioglycollic acid increased the response of *L. leichmannii* to vitamin B_{12}. The addition of thioglycollic acid to crude liver extracts, however, did not affect the response under the conditions of assay used by us and did not minimise the interference caused by the alkali-stable factor. Aeration and treatment with mild oxidising agents likewise failed to affect the assay. It has been shown[17] that vitamin B_{12a} can be converted into vitamin B_{12} by the addition of cyanide to the aqueous solution, whilst it has also been suggested[18] that in liver extracts some or all of the vitamin B_{12} may be present in the form of a complex which is broken down by the addition of cyanide. With the possibility in mind that the alkali-stable substance present in crude liver extracts might be another form of vitamin B_{12} or might be a vitamin B_{12} conjugate, the effect of adding different concentrations of cyanide was investigated; no obvious effect could be detected.

Separation of Vitamin B_{12} from the alkali-stable factor. The first attempts to separate vitamin B_{12} from the alkali-stable factor were made with columns of ion-exchange resin. Columns of Amberlite IRC50, buffered at *p*H 5, 6, 7 and 8, were developed with 0·1N sodium hydroxide, but both factors ran quickly through the columns without any evident separation of one from the other. Similar results were obtained with columns of Amberlite IR400 and Amberlite IR100 buffered at *p*H 3 and 4 and with columns of Zeokarb 215 and 216 developed with 0·1N hydrochloric acid, although the eluates from the Zeokarb 215 columns gave larger vitamin B_{12} zones than did the original extracts.

Next, the extracts were acidified to *p*H 2 and extracted twice with an equal volume of 88 per cent. phenol. The phenol extracts were shaken with water and ether and the aqueous solutions tested. Again, larger vitamin B_{12} zones were obtained than were given by the original extracts, although some alkali-stable factor was probably still present.

Finally a method suggested to us by Peeler and Norris[12] was tried and was found to separate vitamin B_{12} completely from the alkali-stable factor. A 30 per cent. solution of phenol in *n*-butanol was shaken with a 2 per cent. solution of glacial acetic acid adjusted to *p*H 2·0 with hydrochloric acid and the two phases were allowed to separate. 5 ml. of the liver extract was diluted to 100 ml. with the aqueous phase of the equilibrium mixture and the *p*H maintained at 2·0. The solution was then shaken with an equal volume of the alcohol phase of the equilibrium mixture, and the resulting aqueous phase was separated and extracted another seven times with the alcohol-phenol mixture. The residual aqueous phase was shaken with ether to remove phenol and butanol, and then autoclaved at *p*H 1 to destroy thymidine. When assayed by means of *L. leichmannii* or *E. coli* it was found to contain no detectable amount of vitamin B_{12} but with both organisms it gave a large zone of stimulation that was still present after the solution had been boiled at *p*H 10 for 30 minutes. The

combined alcohol-phenol extracts were extracted with water in presence of ether and the aqueous phase evaporated to small volume. The resulting concentrate when assayed gave a zone of stimulation frequently greater than that produced by the original extract, and the zone disappeared completely on alkaline hydrolysis.

Recommended Procedure

In assaying crude liver extracts for vitamin B_{12} by the *L. leichmannii* or *E. coli* cup-plate methods, it is essential to repeat the assay after the extract has been boiled at *p*H 10 for 30 minutes. If a zone of stimulation similar to that given by vitamin B_{12} is still present, then it is recommended that an extraction with phenol-butanol be carried out as described above, and the concentrated extract assayed in the usual way.

Discussion

Although, so far as we are aware, this is the first publication in which attention has been called to the presence in liver extracts of an alkali-stable factor (other than deoxyribosides) that interferes with the microbiological assay of vitamin B_{12}, and in which a method of separating it from vitamin B_{12} has been suggested, there have been a number of references to such substances in earlier publications.

Thus Combs *et al.*[19] reported the existence in a liver concentrate of factors that promoted early growth in chicks and stimulated the growth of *L. leichmannii*. It was shown subsequently[20] that one or other of these factors could replace vitamin B_{12} in the metabolism of *L. leichmannii* and could be differentiated from vitamin B_{12} either by paper chromatography or by treatment with alkali. The new factor stimulated *L. leichmannii* in the early stages of the assay but prolonged incubation eliminated the need for the factor.[21] An alkali-stable factor was also found to be present in alfalfa, an observation confirmed by Bickoff *et al.*[22] Cronheim and Dannenburg[23] have also reported that the "vitamin B_{12}" activity of liver extracts as determined by *L. leichmannii* assays is not completely destroyed by treatment with alkali.

The interfering factor now reported by us appears to be different from that described by Peeler and Norris.[21] Whereas our factor appears to be present only in crude extracts and not in refined extracts Peeler and Norris isolated their factor from 15 unit U.S.P. liver extracts. Again, our factor appears to stimulate the growth of *L. leichmannii* independently of the presence of vitamin B_{12}, whereas Peeler and Norris had to use a dilute inoculum and a short period of incubation to differentiate their factor from vitamin B_{12}; indeed the factor appeared to be synthesised by *L. leichmannii* in the later stages of incubation.

Summary

1. Cup-plate methods for the microbiological assay of vitamin B_{12} with *Lactobacillus leichmannii* and a mutant of *Escherichia coli* are described. The results obtained by the two methods were in reasonably good agreement when applied to most commercial liver extracts.

2. Some crude liver extracts produced several zones of stimulation with both test organisms. One of these zones appeared to be caused by an alkali-stable substance which formed a zone near the vitamin B_{12} zone on paper chromatography. A method for separating this factor from vitamin B_{12} is described.

3. Some evidence was obtained that certain crude liver extracts also contain a substance that interferes with the response of the *E. coli* mutant to vitamin B_{12}.

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