VITAMIN B_{12}

III. THE ASSAY OF VITAMIN B_{12}

by

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In the two preceding papers of this series[1,2] a description has been given of the reaction with KCN of various derivatives of vitamin B_{12}, furtheron designated in this paper as "B_{12}". It has been shown that this reaction, both with B_{12b} and the one or several forms in which the active substance occurs in the liver (provided certain specified conditions were maintained), always leads to the formation of a substance, indistinguishable from B_{12} itself by its spectrum and by paper partition chromatography. That this substance actually is identical with B_{12} has been lately demonstrated both by BRINK *et al.*[3] and by our group[4], though we arrived at this conclusion along quite different lines from these investigators.

The reaction with KCN allows the assay of liver-extracts, which do not contain unduly large amounts of coloured contaminations. Under the influence of KCN, not only a colorimetric determination becomes possible, but also the chromatography of the extracts, as a preparatory step to remove other interfering colours. On the other hand without KCN, chromatography frequently gives rise to several, instead of to one single red band.

The object of this paper is to describe this assay, and to compare the results with those obtained with microbiological methods. Both *L. lactis* Dorner and *L. Leichmannii* were used, the first one in the "cup plate" assay described by CUTHBERTSON[5] and with the turbidimetric method, the second one with the turbidimetric method only.

MATERIAL AND METHODS

Material

The liver fractions used for the chemical determinations were all prepared by N.V. Organon. They contained about 0.2% of B_{12} and mostly brownish and yellow impurities in amounts sufficient to give the powders a pink to buff coloured appearance.

Some samples of commercial products, obtained in the open market, and some very crude liver-extracts were included in the microbiological assays. The activity of these products has not been

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checked clinically by us, and in the majority of the cases it was beyond the scope of the chemical assay.

Microbiological methods

a. *Lactobacillus Leichmannii* (*A.T.C.C.* 4797)

The culture was maintained on a medium of skimmed milk with additions of tomato juice and yeast extract and was transferred every 15 days.

The medium for the microbiological assay was that of SKEGGS *et al.*[6] with the addition of thioglycollic acid. This medium, filled in test tubes, was autoclaved for six minutes at 108–110° after the B_{12} had been added in accurately measured quantities. No detectable loss of this vitamin by the autoclaving process has been observed.

Each time the culture for the inoculation was obtained by transferring one drop of the skimmed milk culture to 5 ml of the medium containing 5 mγ of B_{12}. It was incubated at 40° for 24 hours. After four washings with 20 ml 0.9% saline, the bacteria suspension was adjusted turbidimetrically to a constant optical density. This suspension was used for the inoculation of the test tubes for the assay. These tubes were then maintained at 40° either in the air for 48 hours or in CO_2 containing jars for 24 hours. For each assay a series in duplicate was prepared with 0, 0.05, 0.1, 0.15, 0.2, 0.3 and 0.4 mγ B_{12} per 5 ml. The assay of each sample was performed with three different doses in duplicate runs and repeated after some days. With this scheme, results were obtained with an average error of less than ± 10%[7].

b. *Lactobacillus lactis* Dorner (*Lactobacillus lactis* Orla Jensen, BERGEY *et al.*, strain 1175[8])

L. lactis Dorner, furtheron designated as L.L.D., is weekly transferred on milk (10% yeast-autolysate) and stored at 4°. Daily transfers on basal medium (0.05 μg B_{12}/l), incubated for 24 hours at 40°, are used as an inoculum. Incubation temperature 40°.

Culture medium	Amount for 1000 ml (double strength)
A. sodium acetate	12 g
casein, acid hydrolysed	6 g
enzymatic digest of casein, norit treated	0.3 g
salt mixtures A and B, according to TEPLY AND ELVEHJEM[9], each	10 ml
fumaric acid	500 mg
DL-tryptophane	100 mg
DL-methionine, L-cystine, DL-alanine, each	50 mg
adenine sulphate, guanine hydrochloride, uracil, each	20 mg
xanthine	8 mg
B. oxaloacetate	500 mg
C. glucose	3 g
D-calcium pantothenate, pyridoxine, riboflavin, niacin and thiamine hydrochloride, each	400 μg
p-aminobenzoic acid, biotin, pteroylglutamic acid, each	10 μg

The substances under A are dissolved in 800 ml aq. dest., the medium is adjusted electrometrically to pH 6.9–7, heated until a precipitate flocculates, and then filtered. The oxaloacetate is added and the solution is sterilised through a sintered glassfilter. The volume is brought to 1000 ml with a sterile solution containing C.

Cup plate assay

10 ml of a 24 hours culture of L.L.D. is centrifuged and washed twice with the same amount of basal medium; finally the cells are resuspended in 10 ml basal medium. A mixture of 17.5 ml of basal medium and 17.5 ml 3% Bacto agar in water is made (at 50° C), inoculated with 0.4 ml cell-suspension, and poured into a perfectly flat Petri dish. Five 14 mm holes are punched in each plate.

Freshly prepared solutions of B_{12} in sterile distilled water, containing 0.018, 0.055, 0.166, 0.5 and 1.5 μg B_{12} per ml are used. The unknowns are diluted to the expected potency in the same way.

Each cup is filled with two standard droplets (0.06 ml) of the standard or unknown.

Five cups (on five different plates) are used for each dilution or 25 cups for each sample. The plates are incubated for about 24 hours at 40°. Subsequently the diameter of each growth-zone is measured in three different directions, and the mean of the three measurements recorded.

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Calculation of results

As the relationship between the growth zone and the logarithm of the concentration was linear, the regression coefficient of the log dose-response curve of each sample was computed with the method of least squares, and from the regression coefficients of standard and unknown and the mean responses the amount of B_{12} in the unknown was calculated.

Tube assay

Dilutions of standard or unknown are added to 5 ml of double strength medium in test tubes, the volume is made up to 10 ml and the tubes closed with metal caps. The B_{12} standard is used in sterile solution, stored in ampoules, containing 15 μg pure B_{12} per ml. This solution is diluted to give a final concentration of $6 \cdot 10^{-5}$ μg B_{12} per ml.

0, 1.5, 2, 2.5 and 3 ml aliquots of the standard B_{12} solution are added to a series of tubes, 4 replicate tubes being used at each level of B_{12}.

The unknowns are diluted according to the anticipated potency and aliquots are added in the same way as the standard B_{12} solution. After the addition of standard, unknown, or water, each tube is inoculated with one droplet of a 1:20 dilution of the unwashed 20 hours subculture of L.L.D. The tubes are then shaken to ensure complete mixing. Usually, growth is satisfactory after 14 hours (40°), but an incubation of 16–17 hours is used to ensure maximal differentiation.

After incubation, the bacterial growth is measured turbidimetrically in a Moll extinctometer.

The extinctometer reading is plotted against the concentration of B_{12}/ml. This graph is employed to calculate the B_{12} content of the unknown.

Colorimetric assay

Principle. By incubation with KCN at p_H 5–6, all B_{12} compounds from the liver-extract are converted to B_{12}. The solution can now be chromatographed over neutral Al_2O_3, giving only one broad red band. After elution the colour can be read at 548 mμ.

Reagents

a. *Aluminium oxide.* One kg Al_2O_3 according to BROCKMANN is suspended in 2 l of water. While stirring the suspension is neutralized first with glacial, later on with 8% acetic acid until green to bromothymol blue (external indicator).

The acidity of the solution should remain unchanged in the course of 10 minutes, otherwise some more acetic acid should be added.

The precipitate is separated by decantation and washed several times with tap water, leaving the finest material in the water. To remove all cloudiness (which may seriously interfere with the colorimetric determination), the Al_2O_3 is poured into a large column and washed until the outflow is sparklingly clear. This process may have to be repeated before a sample column of 2.5 g, as used in the analysis, gives a clear outflow. Finally the Al_2O_3 is first dried in air and subsequently at 105–110°.

The fineness of the powder does not perceptably affect the results.

b. freshly prepared 0.1% KCN solution.

c. 0.05 *N* NaOH.

d. 0.05 *N* HCl.

Apparatus

a. Chromatography tubes with an internal diameter of 7–7.5 mm, a perforated glass bottom and a glass stopcock.

b. A Beckman spectrophotometer, model DU, or an apparatus with a similar sensitivity.

c. An incubator or waterbath at 50°–52°.

Method

The sample under investigation should contain from 100–500 μg of B_{12} in 0.5–1 ml of water.

To this solution is added 0.5 ml of the freshly prepared KCN solution. The p_H is adjusted with 0.05 *N* acid or alkali to 5.0–6.5 with the aid of Universal indicator paper. This solution is kept in the dark for 3 hours at 52°. It can also be left overnight at room temperature. After all components have thus been converted to B_{12}, the solution is filtered through a column of 2.5 g Al_2O_3. At the bottom of the tube on the perforated disk should be a small paper filter and no cottonwool plug, as the latter strongly retains B_{12}. The length of the column is approximately 7 cm. The volume of the B_{12} solution should be as small as possible, and in transferring this solution to the column the minimum amount of liquid should be used to rinse the walls of the container in order to ensure narrow bands. The chromatogram is developed with water, and the red eluate is collected. This solution usually is faintly alkaline. The p_H is adjusted to 5.0–6.5, and after the volume V has been measured, the solution is left in the dark for ½–1 hour in order to decompose any of the "alkaline

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reaction product" which might have formed (described in the second paper of this series[2]). Subsequently the extinction E is measured in the spectrophotometer at 548 mμ. Using a 1 cm cuvette, the total amount of B_{12} present can be calculated from the formula:

$$\mu g\ B_{12} = \frac{E.V.\ 10^4}{64} + f$$

With the Al_2O_3 used, the factor 64, obtained for the extinction of pure B_{12} solutions could be used, indicating a quantitative recovery, and the correction factor f equalled zero. With other batches of Al_2O_3 one might be less fortunate, having to apply a correction factor on the numerator or f might not be zero.

It should be mentioned that pure B_{12} under identical circumstances is strongly retained on the column. Therefore the process described is clearly a displacement chromatography, the impurities present in the fraction investigated being stronger adsorbed than B_{12}. However, as soon as other chromogenic factors are removed by further purification of these extracts, the necessity to use this technique becomes less imperative as the extracts can be measured without chromatography.

In case comparatively dilute solutions have to be assayed such as those used clinically, the volume should first be brought down considerably. This can best be done by lyophilizing the appropriate quantity of the sample and subsequently dissolving it again in as small a volume of water as possible.

Equally good results can be obtained by careful vacuumdistillation, provided some KCN is added first and the p_H is maintained between 5 and 6.

RESULTS

a. *The influence of* KCN *on the microbiological assay*

In the colorimetric assay, all B_{12} is converted into the cyano-complex. It had to be proved that the conjugates present in the liver-extracts showed, mole for mole, microbiologically the same activity as the cyano-complex, as otherwise no correlation between microbiological and colorimetric determinations could be expected.

At first it appeared as if the cyano-complex showed a greater activity than the compounds, found in the liver-extracts, when measured with the turbidimetric bioassay. Liver-extracts treated with KCN, tend to show a higher value microbiologically than the same without KCN. This effect is clearly demonstrated by the results of the turbidimetric assay, recorded in Table I (the cup plate assay will be discussed in section c).

TABLE I

B_{12} ASSAYS WITH DIFFERENT METHODS. SOLUTIONS NOT STERILE. RESULTS IN μg B_{12}/ml. PREPARATIONS SUITABLE FOR SPECTROPHOTOMETRIC ASSAY AFTER KCN ADDITION WITHOUT CHROMATOGRAPHY

Code	*L. lactis* Dorner				*L. Leichmannii*		Spectrophotometric
	cup plate		turbidimetric		turbidimetric		
	no KCN	with KCN	no KCN	with KCN	no KCN	with KCN	
B_1	46.4	34.4	17.3	42.7	18.3	33.0	35.1
B_2	21.33	7.5	6	16.9	17.3	16.1	16.4
B_3	18.4	14.8			22.3	36.1	37.4
82 II	63.9	31.8	8.1	14.4	10.7	25.2	
82 II					21.6	34.4	39.8
5663	44.6	18.6	13.7	13.7	15.8	22.7	21.6
5875	34.1	15.6	10.4	20.1	11.8	29.8	29.4
6468	47.4	35.3	12.3	31.6	15.6	31.2	30.6

The difference could be traced to the greater stability of the cyano-complex in the

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following way. A series of 3 samples were assayed with and without KCN. There was a lapse of time of 29 days between the preparation of the solutions and the actual assay. The results are reported in Table II. The results of the KCN-containing samples were all higher than those without KCN, and confirmed the spectrophotometric determination. After a total of 111 days the tests were repeated. The difference between the samples with and without KCN was considerably greater than in the first assay. Finally KCN was added to those solutions which originally did not contain it and the assay repeated the next day, but no increase in activity was observed. KCN therefore has a stabilizing and not an activating effect.

TABLE II

INFLUENCE OF KCN ON ASSAY RESULTS. SOLUTIONS NOT STERILE. RESULTS IN μg B_{12}/ml. ASSAYS WITH *L.Leichmannii*. PREPARATIONS SUITABLE FOR SPECTROPHOTOMETRIC ASSAY AFTER KCN ADDITION WITHOUT CHROMATOGRAPHY

Code	Spectro-photometric	After 29 days		After 111 days		
		solution A (no KCN)	solution B (KCN)	solution A		solution B (KCN)
				no KCN	+ KCN before assay	
5663	21.6	15.8	22.7	9.7	9.6	25.5
5875	29.4	11.8	29.8	7.9	8.2	31.3
6468	30.6	15.6	31.2	9.4	10.2	33.5

It was observed that the KCN effect was absent when the assay samples were sterile. It is therefore assumed that the stabilizing action of KCN is due to the greater stability of the cyano-complex against bacterial attack in comparison to the other modifications of B_{12}.

A difference in stability between B_{12} in liver-extracts and the crystalline substance has also been described by HOFFMANN *et al.*[10]

From Tables I and II it appears that the values of the microbiological assay do not exceed those of the colorimetric determination within the error of the experiment. The latter determination was always performed immediately after the solutions had been made. Bacterial interaction with the B_{12} was therefore excluded during the chemical assay.

The values obtained for samples, without KCN (columns 2, 4, 6 in Table I) are without any real meaning, none of these samples being strictly sterile. They are included to show how great the destruction owing to the contamination by bacteria may be, notwithstanding the presence of toluene, which was added as a routine.

b. *The correlation between microbiological and colorimetric assay*

From the preceding section it follows that reliable microbiological determinations are only possible if decomposition by bacterial attack is avoided.

This can be done either by the addition of KCN to give the stable cyano-complex, by sterile working methods, or by cutting the time between the preparation of the solution and the actual assay sufficiently short as to give infections no time to develop, and preferably by a combination of all these precautions. As for technical reasons the time factor was beyond our control, only those assays with KCN or with sterile solutions

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can be relied upon and be used for the comparison between the two types of assays.

In Table I the solutions mentioned were suitable for colorimetric assay without the aid of a chromatographic separation from coloured impurities. They serve to demonstrate that, if the theoretical extinction of pure B_{12} is used in the formula, a fair agreement is observed between the assays with L.L.D. and *L. Leichmannii* and the colorimetric determination.

In less pure samples, which first have to undergo chromatographic purification the correspondence between the different assays is equally satisfactory, as appears from Table III, thus proving that no activity is left on the column.

TABLE III

B_{12} ASSAYS OF LIVER FRACTIONS. RESULTS IN μg B_{12} PER 100 mg POWDER. CHROMATOGRAPHED FOR SPECTROPHOTOMETRIC ASSAY

Code	439	440	441	442	443	444	445	446
Spectrophotometric	315	385	311	342	294	327* 310	407	342
L. Leichmannii	338	362	294	347 322	286	305	408	366

Mean difference: $-0.59\% \pm 1.8\%$

* Results obtained with two different batches of Al_2O_3

On the other hand, in all cases mentioned in Table IV, the chemical determination has been made on solutions, containing approximately 10 μg B_{12} per ml, which were concentrated at low temperature or lyophilized before the chromatography. These solutions were all sterile.

TABLE IV

B_{12} ASSAYS WITH DIFFERENT METHODS. ALL SOLUTIONS STERILE AND WITHOUT KCN. RESULTS IN μg B_{12}/ml. SOLUTIONS CHROMATOGRAPHED FOR SPECTROPHOTOMETRIC ASSAY

Code	*L. lactis* Dorner		*L. Leichmannii* turbidimetric	Spectrophotometric
	cup plate	turbidimetric		
439	7.5	6.1; 9.6	6.9	6.8
440	13.7	8.6	9.0; 9.8	9.5
NS 441	19.1	9.1	7.7	7.3
NS 442		10.0	9.7; 8.8	8.4
4420			11.1	11.0
44220			10.5	10.2
44237			10.3	10.2
44252			8.9	8.3
443			8.6	8.45
444		10.2	9.0	8.1
10010			10.4	10.4
100152			9.6	9.8
N 442			9.2; 8.8	7.7
N 441			7.9	7.0

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The average result of the spectrophotometric determination falls 4.5% below that of the *L. Leichmannii* assay. The standard error of the percentage deviations of the latter assay compared to the first, is 5.2. A correction factor of 1.045 in the numerator of the formula for the calculation of the B_{12} content from the extinction is therefore indicated to bring the two series of observations in agreement. This correction might be due to losses occurring during the distillation, or to the presence of a preservative (Nipagin) in the original solutions.

c. *The cup plate assay*

In our experience the cup plate assay is easier to handle than the two other microbiological methods described in this paper. The method usually works and gives a result, but unfortunately this result must be considered with suspicion.

The reproducibility of the cup plate assay is satisfactory if strictly comparable samples are considered. With the technique described a series of 11 assays was made of crystalline B_{12} as unknown against the same substance as standard. A standard error of $\pm$ 2.8% for a determination with 25 cups for standard and for unknown was calculated. According to FOSTER *et al.*[11] the standard error for a series of 3 cups both for standard and unknown is approximately $\pm$ 10%.

However, if less pure samples are compared with a crystalline standard, another complication arises. In a series of 36 samples, containing about 0.2–0.4% B_{12} calculated on the dry substance, the regression coefficient of the log dose-response curve has been compared to that of crystalline B_{12}. This regression coefficient has a value varying from 2.0 to 2.5 if the response is expressed in mm. If, for each assay individually, the ratio between the regression coefficients for standard and unknown is calculated, its mean value for all 36 assays is 1.09 $\pm$ 0.016. With the pure samples the mean for 11 assays is 0.987 $\pm$ 0.016.

The highly significant difference in slope may not be apparent in every single assay, but it cannot be due to one or two exceptional results in this series, as the values for the ratio show a normal distribution. Even with the apparently small ratio of the slopes of 1.09, a difference in result of 20% is quite well possible by varying the response level at which unknown and standard are compared.

From Tables I, IV and V it is evident that the cup plate assay, whether used for samples with or without KCN, may give results strongly deviating from the other methods. The effect of KCN is often reversed, the samples without it showing the highest values.

In the cup plate the presence of thymidine in the unknown does not interfere with the B_{12} assay in the same way as it does in the turbidimetric method, and therefore the cup plate should be more specific. Yet in crude preparations, the results are usually much higher than those of the turbidimetric assay. This observation can best be illustrated by the data of Table V. It should be borne in mind that the evaluation of the activity is dependent on the level at which the curves for standard and unknown are compared, as they do not run parallel.

DISCUSSION

As yet, a definite statement about the merits of any microbiological B_{12} assay is more than can be given. All these assays are still quite tricky and it appears from the

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TABLE V

B_{12} ASSAY OF LIVER-EXTRACTS OF DIFFERENT ORIGIN. RESULTS IN μg B_{12}/ml

Code*	Cup plate L.L.D.	Turbidimetric *L. Leichmannii*
L 614	1.8	1.5
L 619	4.6	1.5
L 23	vague zone	1.6
L 25	5.2	1.6
A	5.1	2.2
B	10.3	7.3
C	thymidine + 1	0.8
D	44.4	20.5
E	3.8	1.0
F	6.2	1.8

* Samples L . . . . . are 60% alcoholic extracts from liver
Samples A, B, etc. are products obtained in the open market

literature that many authors had difficulties, in particular with the L.L.D., which they tried to overcome in various ways.

SHAW[12] observed a decreasing sensitivity to B_{12} of his L.L.D. strain. MARY SHORB AND BRIGGS[13] also found their assay less satisfactory after several transfers of their culture. GREENE *et al.*[14] described an effect of the amount of air dissolved in the culture medium which was quite considerable. WELCH AND WILSON[15] pointed out that ascorbic acid and other reducing substances in a medium containing a tryptic casein digest, would give growth, in particular after autoclaving. KODITSCHEK *et al.*[16] found an influence of the presence of CO_2 and oxidizing agents.

The complaints about *L. Leichmannii* are less frequent. SKEGGS *et al.*[5] observed that the presence of air had a certain influence on the result. The effect of reducing substances is also present, according to STOKSTAD *et al.*[10,17]

It is obvious, that even apparently minor variations in the technique may greatly influence the results and the reproducibility.

The turbidimetric methods appear to be somewhat more difficult to control than the cup plate assay, and of these two, the one using *L. Leichmannii* appears to be more suitable than that with L.L.D. This evaluation of necessity bears a subjective character. It is quite well possible that other investigators, with a slightly different method come to other conclusions.

The cup plate assay with L.L.D. has not been very satisfactory in our hands, giving only a very approximate idea of the B_{12} content. In the hands of others it may prove to be more specific and more reliable, but this proof cannot be given by simply stressing the small standard deviation. In order to make it acceptable it would also be necessary to show that, with another technique than the one employed, the slope of the dose-response curve is independent of the purity of the samples under investigation. That this slope may vary with the purity is an effect, which, after all, can be anticipated. In the test tube assay the number of variables is already quite large. In the cup plate assay some additional factors are also involved. One of the essential conditions is the equality of the diffusion coefficients of standard and unknown. There is no reason to assume without further proof that the diffusion coefficient of the B_{12} complex in liver (probably linked to a polypeptide or protein[2]) will be equal to that of the crystalline

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cyano-complex used as a standard. The data of Table I of the results of the cup plate assay might indicate that the B_{12}-protein complex has a greater diffusion coefficient than the cyano-complex. Another possible explanation is the different rate of adsorptiont hese complexes might have on the agar gel employed in the plate. Further more, in crude or in electrolyte containing preparations with a high osmotic pressure, there may be expected a flow of solvent towards the cup, interfering with the diffusion.

All these possible sources of error, added to our unsatisfactory results in practice, lead us to believe that the cup plate in the form as outlined in this paper, is unsuitable for accurate assays. The misleading results the cup plate may give, have also been described by HARTLEY *et al.*[18]

Of the assays in a liquid medium, the correlation between that with *L. Leichmannii* and the spectrophotometric method is the most satisfactory. As the latter is based on the extinction of pure B_{12} this means that the forms, in which this vitamin occurs in the liverextracts, are microbiologically just as active as the equivalent amount of the cyano-complex within the limits of the experimental error. We also observed that B_{12b} shows the same activity, in agreement with the results of PIERCE *et al.*[19]

In transforming these complexes to the cyano-complex for the photometric determination, no change in activity is achieved. An apparent increase, which may be observed microbiologically is due to the stabilizing action of the KCN.

The combination of the microbiological and the spectrophotometric assay gives as yet the most reliable results. It has not be ascertained in how far the spectrophotometric method can be used in extracts of different origin, or prepared in a very different way.

There might be complexes of B_{12} in which it is so firmly bound that it cannot be freed by the addition of KCN, yet shows its full clinical activity. The factor, occuring in gastric juice, described by TERNBERG AND EAKIN[20], which makes B_{12} inaccessible to L.L.D., might interfere, if it were present. In the refined liver-extracts which were the subject of this investigation, no indications of the presence in appreciable amounts of such firmly bound complexes were obtained.

The colorimetric assay here proposed, is at first sight definitely more limited in its scope than that, proposed by FANTES, IRELAND, AND GREEN[21] or by BOXER AND RICKARDS[22]. Yet, its speed and simplicity make it very useful for routine determinations in those cases, which fall within its range of possibilities. Before such a routine determination is initiated with material of different origin as that used in this work, a check on the results by means of a series of microbiological assays is required.

SUMMARY

1. After the addition of KCN, purified liver-extracts can be chromatographed on Al_2O_3, the vitamin B_{12} being collected as one single band which can be measured spectrophotometrically.

2. The values, thus obtained, agree satisfactorily with those of the *L. Leichmannii* and a little less with the *L. lactis* Dorner assay. both in liquid medium.

3. In our hands the cup plate assay with *L. lactis* Dorner proved less satisfactory, the slope of the dose-response curve being dependent on the purity of the sample under investigation.

RÉSUMÉ

1. La chromatographie sur oxyde d'alumine d'extraits de foie purifiés et traités au cyanure de potassium, permet d'isoler la vitamine B_{12} et d'en déterminer le taux par spectrophotométrie.

2. L'accord entre les résultats fournis par la méthode spectrophotométrique et la mesure micro-

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biologique est satisfaisant dans les essais en milieu liquide avec *L. Leichmannii* et l'est un peu moins dans les essais avec *L. lactis* Dorner.

3. L'essai microbiologique sur gélose (cup plate) avec *L. lactis* Dorner a donné des résultats moins satisfaisants, la pente du tracé étant fonction du degré de pureté de l'échantillon soumis à l'essai.

ZUSAMMENFASSUNG

1. Gereinigte Leberextrakte können nach Zufügen von KCN an Al_2O_3 chromatografiert werden; Vitamin B_{12} wird als einzelnen Band erhalten, das spektrophotometrisch gemessen werden kann.

2. Die so erhaltenen Werte stimmen in befriedigender Weise mit denen überein, welche mit *L. Leichmannii* erhalten werden, und etwas weniger mit jenen von *L. lactis* Dorner, beide Male in flüssigem Medium bestimmt.

3. In unseren Händen erwies sich der "cup plate assay" mit *L. lactis* Dorner als weniger befriedigend, indem die Neigung der Dosis-Wirkung Kurve von der Reinheit des untersuchten Präparates abhängt.

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