STUDIES ON GROWTH PRINCIPLES IN LIVER

I. Separation of Cyano-Cobalamine from other Growth Factors and its Estimation by Different Methods

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Introduction

Recent studies on the active principles of liver indicate that it might contain, amongst others, vitamin B_{12} complex (Robinson *et al.*, 1952), nucleotides (Hurlbert *et al.*, 1954), nucleosides (Orunesu, 1953), and similar other factors. In 1949 Winsten and Eigen analysed various liver preparations possessing anti-pernicious anaemia or animal protein factor activity, and detected the existence of at least six entities capable of supporting the growth of *L. leichmanii* in the vitamin B_{12} deficient medium, but these entities have not yet been fully characterised. Shive (1948) suggested the desoxyriboside nature of the alkali-stable bacterial growth factors of liver. Dutta (1955) isolated two such factors and characterised them as a purine and a pyrimidine desoxyriboside.

It was a problem to separate the vitamin B_{12} in liver homogenates from the alkali-stable growth factors, and to estimate the same. Several authors applied microbiological method (Thompson *et al.*, 1950; Harrison *et al.*, 1951) to estimate vitamin B_{12} in liver homogenates. Later Boxer and Rickard (1952) devised an efficient method to estimate the total cyanide of the vitamin B_{12} in liver homogenates. Klaveren *et al.* (1954) reported a method of photometric determination of the vitamin B_{12}, based on the work of Reichstein *et al.* (1951) and Lester and Smith (1952). Rudkin and Taylor (1952) measured the extinction of the purple colour of the dicyano-complex (582 mμ) after having treated the liver extracts for five hours with sodium cyanide at *p*H 9·5–10·0 and extracting the complex with benzyl alcohol at *p*H 11–11·5. Lens *et al.* (1952) reported separation of vitamin B_{12} in liver extracts on aluminium oxide column and estimation by elution and measuring the absorption at 548 mμ.

Liver contains not only cyano-cobalamine but also hydroxo-cobalamine in significant amount. Wijmenga *et al.* (1950) have shown that hydroxo-cobalamine could be easily converted to cyano-cobalamine completely. Exact estimation of the amount of hydroxo-cobalamine in presence of the latter is complex. No microbiological method could serve this purpose, unless they were sharply fractionated into two groups. Boxer and Rickard (1952) suggested the total estimation of cyanide before and after treatment of the substrate with potassium cyanide and thus to calculate the amount of hydroxo-cobalamine. Other colorimetric or photometric method as already referred to could not be modified to serve similarly.

In the present investigation attempt has been made to confirm that besides vitamin B_{12} there might be present some other growth factors in liver homogenates and subsequently to isolate the cyano-cobalamine from those factors and to estimate it; and lastly to estimate hydroxo-cobalamine in liver extracts.

Materials

(*a*) Acetone—purified by distillation in an all-glass distilling set.
(*b*) *n*-Butanol—purified by refluxing with pure sodium hydroxide and zinc dust for six hours and then distilling the solvent thrice.
(*c*) Pyridine—purified by distillation in an all-glass set.
(*d*) Ammonium sulfate (extra pure quality).
(*e*) Potassium dihydrogen phosphate (E. Merck).
(*f*) Cyano-cobalamine (Merck & Co.).
(*g*) Zeo-karb 215 (The Permutit Co., Ltd.).
(*h*) Sodium cyanide (extra pure quality).

Beckman spectrophotometer (Model DU) was used for all absorption analyses. Microbiological assays were done with *E. coli* (Bose, 1955) * and with *L. leichmanii* 313 (Robinson *et al.*, 1952).

Method

Raw liver was proteolysed with papain (B.P.C.) at 45°C. and the residual undigested protein and proteoses were removed at 0°C. by ethanol in presence of Ca^{++} ion. Clear proteolysed solution had a solid content 20%, total N_2 3% and total α-amino nitrogen 1·9%.

Zeo-karb 215 was used to separate the alkali-stable growth factors from amino acids and the vitamin B_{12} group. The adsorbed vitamin could be eluted with aqueous ammonia and estimated.

A paper chromatographic method was developed to separate the cyano-cobalamine from the other growth principles. Whatman No. 1 paper, impregnated with potassium dihydrogen phosphate, was used. The developing solvent was a mixture of *n*-butanol, pyridine, and water. The cyano-cobalamine was eluted with water and estimated either by microbiological or spectrophotometric method.

In order to have a solution richer in the cyano-cobalamine, the proteolysed solution might be extracted with acetone in presence of ammonium sulfate. After removing the acetone the extract became several times richer in vitamin B_{12} content. When this solution was chromatographed, the separated cyano-cobalamine could be eluted and estimated directly by measuring the absorption at 361 mμ (using proper blank eluate).

Experimental

Zeo-karb 215 (3·5 g.) was thoroughly washed with distilled water for about four days and then filled in a glass column (20×0·5 cm.). Then 50 c.c. of dilute hydrochloric acid (3%) was passed (a drop per second) through the column followed by excess of distilled water until the *p*H of the effluent reached about 6·5. The column was then treated with 1% aqueous ammonia solution (50 c.c.) and rewashed with distilled water until *p*H of the effluent was about 7·0. The column was re-treated with 3% hydrochloric acid and freed from the acid with distilled water.

One c.c. of standard cyano-cobalamine solution (50 μg. per c.c.) was passed through the column, followed by 9 c.c. of distilled water. Total effluent, 10 c.c.

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* The principle of the method is based upon the measurement of the growth zones of test organism after 24 hours of incubation. The assay organisms used are (i) *Lact. leichmanii* 313 and (ii) *Esch. coli* 301 (Mutant). The medium for *L. leichmanii* is a new one prepared here, and that for *E. coli* is one modified from the work of Harrison, Lees and Wood. Variable doses of standard vitamin B_{12} solution and that of test solutions are put on the same agar plate and subsequently the zone diameters are measured. Estimation of vitamin B_{12} is done by finding out the log dose ratio between the curves of the standard vitamin B_{12} and the test solutions, which show a parallel response.

(*a*), was collected. The column was washed with distilled water, 20 c.c. (*b*). The column was then eluted with 1·5% aqueous ammonia, 10 c.c. (*c*). The eluate (*c*) was concentrated at low temperature (30°C.) to 5 c.c. volume, when ammonia was removed. Absorption at 361 mμ was recorded. The recovery obtained was more than 98% (case 1, vide Table A in 'Discussion').

Studies on hydroxo-cobalamine (its preparation will be described later) with Zeo-karb 215 produced similar results, measuring the $E_{max.}$ at 351 mμ.

It was noted that dilute aqueous ammonia has no deteriorating effect on the cyano-cobalamine or the hydroxo-cobalamine at room temperature (about 30°C.), even if left for several days.

The resin was then regenerated with hydrochloric acid and washed as before.

Two c.c. of proteolysed liver solution, containing 1·5 μg. vitamin B_{12} per c.c., was similarly passed through the column, followed by 9 c.c. of distilled water, the effluent being 10 c.c. (*d*). The column was washed with 30 c.c. of distilled water and the effluent was 30 c.c. (*e*). The column was then eluted with 1·5% aqueous ammonia, the eluate being 20 c.c., which was concentrated to 2 c.c. (*f*). Estimation of the vitamin B_{12} in the fraction (*f*), according to Boxer and Rickard (1952), gave 2·0 μg. per c.c., while by microbiological method (Bose) 1·5 μg. per c.c. (case 2, vide Table A in 'Discussion'). It was noted in several cases that the former method had a tendency to give higher result than the latter method.

Effluent (*e*) was found to have no growth promoting effect on *L. leichmanii* or *E. coli*. The effluent (*d*) was a colourless clear solution, showing sharp UV-absorption at 260 mμ and having no absorption from 290 mμ to 400 mμ. It stimulated the growth of *L. leichmanii* 313 and its activity was not reduced by heating at *p*H 12·0 (110°C./10 min.). Preliminary report of the analysis of this fraction (*d*) was published elsewhere (Dutta, 1955).

The proteolysed liver solution was then adjuvated so that each c.c. would contain 24 μg. vitamin B_{12}. One c.c. of this adjuvated solution was similarly analysed as above and found to contain 23·8 μg. cyano-cobalamine (Robinson *et al.*, 1952) (case 3, vide Table A in 'Discussion').

Paper chromatography was then successfully applied to separate the cyano-cobalamine from other growth factors in liver homogenates. Whatman No. 1 (chromatographic paper) was washed with 1% potassium dihydrogen phosphate solution and dried at 40°C. Two developing solvents were found effective—(I) a mixture of *n*-butanol, ethanol, and distilled water (4: 5: 2·5), and (II) a mixture of *n*-butanol, pyridine, and distilled water (6: 4: 3). In the latter case glass distilled water was used. It was noted that the solvent (II) was preferable and so was used for the following investigations. Development was done at room temperature in darkness for six hours.

Experimental work with standard cyano-cobalamine showed that vitamin B_{12} moved with R_f 0·24 and could be recovered from the paper up to 98%. The alkali-stable growth factors, as isolated by Zeo-karb 215, were also developed on the same chromatogram, when it was noted that they were separated into two groups, one having R_f 0·0 and the other 0·39, and none having R_f 0·24. However, at R_f 0·24 some amino acids seemed to be present after the development of the chromatogram. These amino acids were noted to have no absorption at 361 mμ and no growth stimulating effect on *L. leichmanii* or *E. coli*. All colouring matter remained at the starting zone.

Separation of hydroxo-cobalamine from cyano-cobalamine by the paper chromatogram was also studied. Vitamin B_{12a} (or B_{12b}) was prepared from the cyano-cobalamine thus: an aqueous solution of vitamin B_{12} was acidified with sulfuric acid to have final concentration 0·001N and final volume 10 c.c. with 20 μg. vitamin B_{12} per c.c. It was then exposed to light (two 500 watts projection lamps on both sides of the substrate, which was immersed in a water bath at 25°–30°C.) for two hours, when nitrogen gas, purified according to Boxer and Rickard (1952),

was passed through to drive away the liberated CN^- groups. The *p*H of the solution was then adjusted to 6·0 and the absorption spectrum (from 200 mμ to 600 mμ) was recorded to have $E_{max.}$ at 351 mμ (instead of 361 mμ) and at 525 mμ (instead of 550 mμ) (Wijmenga *et al.*, 1950). Then aliquots of this vitamin B_{12b} solution was chromatographed, parallel to vitamin B_{12}, and it was noted that the former moved much slower, having R_f 0·05 only, while the latter with R_f 0·24. Thus the cyano-cobalamine can be separated completely from vitamin B_{12b} and the alkali-stable factors.

The cyano-cobalamine, thus separated on the paper, could be eluted with water and then estimated, either microbiologically or by optical absorption at 361 mμ, the latter method requiring a higher concentration of the vitamin (at least 10 μg. per c.c. of the eluate).

Proteolysed liver solution (containing 2·24 μg. cyano-cobalamine per c.c.) was chromatographed and the fraction with R_f 0·24 was eluted with water and analysed microbiologically and found to contain 2·10 μg. cyano-cobalamine per c.c. (case 4, vide Table A in 'Discussion').

A method was developed to extract the vitamin from the proteolysed liver solution and thus to have a concentrated solution, which could be chromatographed, the separated vitamin eluted and estimated by measuring the absorption at 361 mμ. With standard cyano-cobalamine it was noted that from aqueous solution the vitamin could be extracted completely with acetone at 75–80% saturation with ammonium sulfate. Moreover it was noted that acetone, saturated with water and ammonium sulfate, had no significant deteriorating effect on the cyano-cobalamine, even at 100°C. for several hours.

A proteolysed liver solution (50 c.c.), adjuvated so as to contain 20 μg. cyano-cobalamine per c.c., was mixed with acetone (25 c.c.) and stirred uniformly, when gradually requisite amount of ammonium sulfate was added. It was then centrifuged and the acetone layer collected. The aqueous layer was further extracted twice more with acetone (15 × 2 c.c.). All the acetone extract was taken together and concentrated at 30°C. to 2·0 c.c. This extract contained some of the alkali-stable growth factors also in addition to the vitamin B_{12}. One c.c. of this solution was treated with Zeo-karb 215 (H-form) and the UV-absorption spectrum of the diluted effluent was recorded (A in Fig. 1). The effluent on being chromatographed showed two zones with R_f 0·0 and 0·39 and both fractions stimulated *L. leichmanii* 313. Then 0·25 c.c. of the above extract was directly chromatographed and the zone with R_f 0·24, parallel to standard vitamin B_{12}, was cut out, eluted with 5 c.c. of distilled water, centrifuged, and its absorption at 361 mμ recorded and each c.c. of the eluate was found to contain 24·2 μg. cyano-cobalamine. The recovery was 121·0 μg. in place of 125·0 μg. (case 5, vide Table A in 'Discussion'). The zones with R_f 0·0 and 0·39 on the chromatogram were detected to contain some alkali-stable growth factors.

The aqueous layer, after the acetone extract, was then passed through a column of Zeo-karb 215 (H-form) and the colourless effluent was collected. The *p*H of the effluent was adjusted to 7·0 with ammonia when flocculent precipitate appeared. It was centrifuged and the clear solution was analysed. Its UV-absorption curve was recorded (B in Fig. 1), it was chromatographed to note its R_f as 0·0 and this fraction had a stimulating effect on *L. leichmanii* like vitamin B_{12}. The flocculent precipitate was washed twice with water and dissolved in 0·1 N hydrochloric acid and its UV-absorption curve was recorded (C in Fig. 1). It had no bacterial growth promoting property.

Attempt was then made to have an idea about the amount of hydroxo-cobalamine that might be present in proteolysed liver solutions. Proteolysed liver solution containing 1·75 μg. vitamin B_{12} per c.c., as estimated by separation on paper chromatogram and assaying by microbiological method, was then treated with sodium cyanide (0·1%), the *p*H being adjusted to 6·0, and ampouled and

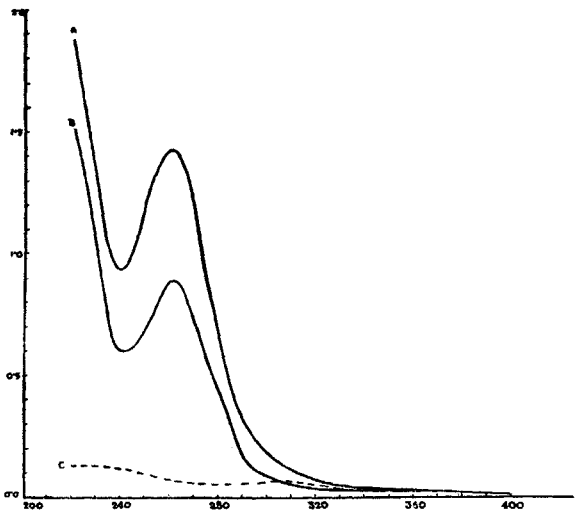

FIG. 1

heated at 52°C. for three hours in darkness and then left in darkness at room temperature for 24 hours. Then 0·08 c.c. of that solution was chromatographed, as before, in darkness and assayed microbiologically (*E. coli.*, Bose) and was found to contain 0·27 μg. cyano-cobalamine. Thus each c.c. seemed to contain 3·37 μg. vitamin B_{12} in place of 1·75 μg. So 1·62 μg. cyano-cobalamine per c.c. was freshly formed on cyanide treatment of the proteolysed solution.

Result

Proteolysed liver solution contained, in addition to the vitamin B_{12}, some vitamin B_{12b} and some alkali-stable growth factors, and the vitamin B_{12} could be separated from those factors and estimated.

Discussion

Several authors have used various methods to estimate the vitamin B_{12} in liver extracts. Direct microbiological method might not offer reliable results owing to the presence of other growth factors, like hydroxo-cobalamine and alkali-stable growth factors. The estimation of CN^- group according to Boxer and Rickard (1952) gave fair results, but it was a rather long and tedious process. The column chromatographic method of Lens *et al.* (1952) could be applied to only those solutions which contained at least 100–500 μg. vitamin B_{12} per c.c. and moreover the method became complex for the stringency on the quality of aluminium oxide. Furthermore it was not applicable to purified liver preparation. The combination of paper chromatography with either microbiological or spectrophotometrical method as shown in the present paper is rather simple and requires no special equipments. By this method liver extracts containing as little as 1-2 μg. cyano-cobalamine or hydroxo-cobalamine per c.c. may be accurately assayed. Moreover the degree of purification of the liver extract does not affect the method in any way.

To study the growth principles in liver by the above method, the liver should be proteolysed and freed from undigested proteins and proteoses under mild

conditions, controlling *p*H, temperature, etc. and giving due attention to the intracellular enzymes, so that the active principles may not be degraded during the preparation of the solution. Concentration of the various fractions was done by vacuum distillation. But freeze-drying is preferable in such cases.

A fairly good recovery of the cyano-cobalamine as shown in this paper would be evident from the results as tabulated below. The results are from an average of five experiments in each case.

TABLE A

Case	Theoretical content of B_{12} (μg.)	Found B_{12} (μg.)	Method	Ref.
1	50·0	48·0	Spectrophotometric	
2	1·5	2·0	CN estimation	Boxer and Rickard (1952).
		1·5	Microbiological	Bose (1955)
3	24·0	23·8	do.	Robinson *et al.* (1952)
4	2·24	2·10	do.	Bose (1955)
5	125·0	121·0	Spectrophotometric	

From the above observations it appears that the presence of a stabler form of vitamin B_{12} as noted by Ramsarma and Shenoy (1955) needs re-examination. Studies on the conversion of the allied substances of vitamin B_{12} with sodium cyanide and their chromatographic separation and subsequent estimation clearly indicate the presence of vitamin B_{12b} in the proteolysed liver solution.

SUMMARY

Raw liver was proteolysed with papain and freed from undigested proteins, etc. It was then treated with activated Zero-karb 215 to separate the vitamin B_{12} from the alkali-stable growth factors. The vitamin B_{12} group was eluted and assayed microbiologically. Paper chromatography was then applied to separate the cyano-cobalamine from hydroxo-cobalamine and the alkali-stable growth factors. The cyano-cobalamine could be eluted from the paper and estimated either by microbiological method or by spectrophotometry. It was also possible to estimate the amount of hydroxo-cobalamine in the proteolysed solution.

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