VITAMIN B_{12} BINDING IN BACTERIAL RIBOSOMES AND OBSERVATION OF A RIBOSOMAL VITAMIN B_{12} BINDING GLYCOPROTEIN[1]

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The present report gives evidence that the ribosomes of Lactobacillus leichmannii and Escherichia coli bind vitamin B_{12}. The latter organism has no requirement for exogenous vitamin B_{12}; the former requires B_{12} or deoxyribosides (Kitay, McNutt and Snell, 1950). Previous studies have shown that L. leichmannii binds vitamin B_{12} in considerably larger quantities than are needed for maximal growth. The binding mechanism is apparently independent of cellular metabolic processes (Kashket and Tave, 1960). The experiments to be described also indicate that L. leichmannii ribosomes contain a vitamin B_{12} binding glycoprotein-like material. It has not yet been determined whether a similar protein exists in E. coli ribosomes.

Vitamin $B_{12}Co^{60}$ Binding in Lactobacillus leichmannii Ribosomes -- L. leichmannii (ATCC 7830) were cultivated for 10 to 16 hours at 37° in Difco Assay Medium USP[2] plus 0.05 to 0.15 mμg. vitamin $B_{12}Co^{60}$ per ml. Cells were harvested by centrifugation, washed twice in 0.9 per cent NaCl solution and once in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer that had been adjusted to pH 7.2 with 0.2 M succinic acid and to which 0.1 M magnesium acetate had been added to a Mg^{++} concentration of

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[2] Composition of the medium is given in the Difco Manual, p. 221, 9th ed., Difco Laboratories, Detroit, Michigan.

TABLE I

Distribution of Radioactivity in the Centrifuge Tube after 105,000 x g Centrifugation of Preparations from $B_{12}Co^{60}$-grown Lactobacillus leichmannii.

Expt.[+]	Fraction[++]	Per cent of total radio-activity	Per cent of total radio-activity per ml.	$\frac{D_{280}}{D_{260}}$	Per cent of total pentose	Cpm. per mg. of pentose
	ml.					
1	3.0	1.6	0.5		9.7	556
	2.0	1.0	0.5		5.9	573
	2.0	1.1	0.6		5.9	634
	1.2	39.1	32.6		33.4	4,010
	Pellet	57.2	--		45.1	4,330
		100.0			100.0	
2	3.0	4.5	1.5	0.56		
Sucrose	3.0	5.4	1.8	0.53		
	3.0	9.9	3.3	0.52		
	1.2	9.2	7.7	0.51		
	Pellet	71.0	--	0.52		
		100.0				
3	2.0	3.9	2.0	0.79		
DOC	2.0	3.6	1.8	0.77		
	2.0	4.9	2.5	0.74		
	2.0	6.0	3.0	0.79		
	1.9	9.0	4.7	0.77		
	Pellet	72.6	--	0.47		
		100.0				
4	2.0	13.9	7.0	0.74	0.1	
CsCl	2.0	16.8	8.4	0.80	0.1	
	2.0	22.9	11.5	0.83	0.3	
	2.0	25.6	12.8	0.76	0.3	
	2.2	16.0	7.3	0.58	1.2	
	Pellet	4.8	--	0.48	98.0	
		100.0			100.0	

[+] In Experiment 1, an initial extract was centrifuged. Pellets of the type obtained in Experiment 1 were recentrifuged in 50 per cent sucrose in Experiment 2; in 1 per cent sodium deoxycholate in 5 x 10^{-3} M Mg^{++} in Experiment 3; and in 40 per cent CsCl in 0.01 M Mg^{++} in Experiment 4. Other details are in the text.

[++] Refers to the volume and position of the sample in the centrifuge tube. Thus, in Experiment 1, the upper 3.0 ml. in the tube was sampled first, then the next 2.0 ml., etc.

5×10^{-3}M. Washed cells were sonicated and centrifuged at 25,000 x g for 30 minutes to remove unbroken cells and debris.

Table I presents results of typical experiments. In Experiment 1, a 25,000 x g supernatant fraction was centrifuged for 2.5 hours at 105,000 x g. Of the total extract radioactivity, 57.2 per cent was recovered in the pellet and 39.1 per cent in the turbid supernatant layer immediately above the pellet. The latter presumably contained unsedimented ribosomal particles. The two fractions contained 96.3 per cent of the total radioactivity and 78.5 per cent of the total pentose of the extract. The specific activity of the fractions (per cent of total radioactivity per ml. or cpm. per mg. pentose) was highest in these fractions. The bulk of the pentose of the upper 7 ml. presumably represents soluble RNA so that only 14 per cent of the pentose remaining in the 3 upper fractions would have to be of ribosomal origin to account for all the radioactivity found in these fractions.

In the remaining experiments, pellets similar to that obtained in Experiment 1 were variously resuspended and recentrifuged. In Experiment 2, centrifugation in 50 per cent sucrose in 5×10^{-3}M Mg^{++} buffer at 105,000 x g for 12 hours produced a pellet containing 71 per cent of the total radioactivity. The distribution of the remaining radioactivity and the D_{280}/D_{260} ratios suggests incomplete sedimentation of ribosomes with continued association of the $B_{12}Co^{60}$ with the ribosomal particles. Similarly, centrifugation of $B_{12}Co^{60}$-labeled ribosomes in 1 per cent sodium deoxycholate in 5×10^{-3}M Mg^{++} buffer at 105,000 x g for 5 hours (Experiment 3) deposited 72.6 per cent of the radioactivity in the pellet. However, centrifugation in 40 per cent CsCl in 5×10^{-3}M or 1×10^{-2}M Mg^{++} buffer (Experiment 4) removed a protein component bearing most of the radioactivity from the ribosomes, as indicated by the high D_{280}/D_{260} ratios and specific radioactivities of the middle layers of the supernatant fraction and by the fact that the pellet contained 98 per cent of the total ribose but only 4.8

per cent of the total radioactivity.

Chromatography of $B_{12}Co^{60}$-containing ribosomes on DEAE cellulose with a linear NaCl gradient revealed a sharp ribosomal peak containing almost all the radioactivity[3]. It is interesting that although trans-deoxynucleosidase occurred in the ribosomal pellet obtained after sonication, the enzyme activity was cleanly separated from the ribosomal peak by cellulose chromatography[4].

Isolation of a Ribosomal Glycoprotein in Lactobacillus leichmannii -- Glycoprotein was prepared from L. leichmannii ribosomes with perchloric and phosphotungstic acids (Winzler et al., 1948). Glycoprotein prepared from $B_{12}Co^{60}$-labeled ribosomes contained approximately 70 per cent of the ribosomal radioactivity. Glycoprotein from unlabeled ribosomes rapidly bound free $B_{12}Co^{60}$ making it non-dialyzable. Preliminary results indicate the presence of glucose and galactose and a small amount of ribose in the glycoprotein preparation; purines and pyrimidines were absent. The ribosomal glycoprotein was subjected to paper electrophoresis in acetate buffer at pH 4.5. Vitamin B_{12} activity (as determined by bioautography) remained nearer the origin than a reference sample of free vitamin B_{12} which migrated toward the cathode. Serum B_{12}-binding protein has been shown to migrate freely toward the anode under similar conditions (Winzler et al., 1948; Miller and Sullivan, 1959), suggesting that the ribosomal preparation differs from the serum glycoprotein.

Vitamin B_{12} in Purified Ribosomes -- E. coli, strain W, were grown in a minimal salts medium plus glucose and 0.02 mμg. $B_{12}Co^{60}$ per ml. Cell extracts were prepared as for L. leichmannii. About 10 per cent

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[3] S. Kashket, I. B. Weinstein and W. S. Beck, to be published.

[4] M. Levin and W. S. Beck, to be published.

of the total radioactivity of the extracts was found in the ribosomal pellet, but further centrifugation of the ribosomes in 50 per cent sucrose showed that the radioactivity was actually associated with the ribosomes and not with contaminants of the ribosomal pellet. Purified preparations of E. coli ribosomes with sedimentation constants 50 S and 70 S (Tissières et al., 1959)[5] were then assayed microbiologically for vitamin B_{12} using the L. leichmannii method[6]. Both samples were found to contain large amounts of vitamin B_{12}; the 70 S preparation contained 1.0 mμg. of vitamin B_{12} per 4.2 mg. of ribosomal protein or per 6.5 mg. of nucleic acid.

Preliminary purification of L. leichmannii ribosomes yielded 30 S particles which retained $B_{12}Co^{60}$. Preparations of 50 S particles have not yet been assayed.

Discussion -- Association of vitamin B_{12} with mammalian liver microsomes has been previously reported (Swenseid et al., 1951; Wagle et al., 1958). The present results indicate that the vitamin is bound to ribosomal ribonucleoprotein and not to deoxycholate soluble material or contaminants such as cell wall fragments. The evidence favoring this view is the continued association of the $B_{12}Co^{60}$ with the ribosomal pellet through sucrose and deoxycholate centrifugation, cellulose chromatography and the detection of vitamin B_{12} in ultracentifugally homogeneous E. coli and L. leichmannii ribosomes. The removal of protein material and $B_{12}Co^{60}$ by CsCl is, therefore, unexpected and may represent an artefact.

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[5] Kindly supplied by Dr. A. Tissières.

[6] Microbiological assays were performed by Miss Corinne Bryant.

References

Kashket, S. and Tave, J. L., Fed. Proc., 19, 416 (1960).

Kitay, E., McNutt, W. S. and Snell, E. E., J. Bact., 59, 727 (1950).

Miller, A. and Sullivan, J. F., J. Clin. Invest., 38, 2135 (1959).

Swenseid, M. D., Bethell, F. H. and Ackerman, W. W., J. Biol. Chem., 190, 791 (1951).

Tissières, A., Watson, J. D., Schlessinger, D. and Hollingworth, B. R., J. Mol. Biol., 1, 221 (1959).

Wagle, S. R., Mehta, R. and Johnson, B. C., J. Biol. Chem., 233, 519 (1958).

Winzler, R. J., Dever, A. W., Mehl, J. W. and Smyth, I. M., J. Clin. Invest. 27, 609 (1948).