COBAMIDE STIMULATION OF THE REDUCTION OF RIBOTIDES TO DEOXYRIBOTIDES IN LACTOBACILLUS LEICHMANNII

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A relationship between vitamin B_{12} (cyano 5,6-dimethylbenzimidazole cobamide) and deoxyribose compounds in the metabolism of Lactobacillus leichmannii was originally established by studies of the growth requirements of this organism (Snell et al., 1948; Wright et al., 1948; Kitay et al., 1949; Kitay et al., 1950). This relationship has been confirmed in several laboratories by studies in which the organism has been grown on a medium containing a ribotide generally labelled with ^{14}C (Wacker et al., 1959; Manson, 1962). In these experiments it was demonstrated that the labelled ribose moiety was incorporated into deoxyribose of DNA (which was examined as derived deoxyribonucleotides or deoxyribonucleosides) only when vitamin B_{12} was present in the growth medium. A similar requirement for the incorporation of ribose-1-^{14}C into DNA of growing cells of L. leichmannii was briefly reported by Dinning (1959).

We have confirmed the last mentioned observation in

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some detail as a preliminary to studies with cell-free extracts. L. leichmannii A.T.C.C. 4797 was grown on a chemically-defined medium which was supplemented with either vitamin B_{12} (0.05 mμg./ml.) or thymidine (8 μg./ml.), and which also contained 25 μC of ribose-1-^{14}C (8.6 μmoles) per litre. The medium contained 2% glucose as carbon source, amino acids, vitamins, purine bases and uracil, Tween 80 and salts. Cells were harvested, washed and lysed and the ribonucleosides from RNA and deoxyribonucleosides from DNA were isolated, purified by chromatography and their radioactivities determined. The results, shown in Table 1, clearly support the contention that in this organism ribose derivatives can be converted to deoxyribose derivatives only by a pathway in which cobamide derivatives are intimately involved.

Table 1

Incorporation of ribose-1-^{14}C into ribosides of RNA and deoxyribosides of DNA by growing cells of L. leichmannii

Supplement to Medium	Riboside	c.p.m./ μmole	Deoxyriboside	c.p.m./ μmole
Vitamin B_{12}	Adenosine	14,100	Deoxyadenosine	11,800
	Cytidine	13,200	Deoxycytidine	13,600
	Guanosine	12,900	Deoxyguanosine	11,200
	Uridine	16,200	Thymidine	15,900
Thymidine	Adenosine	13,700	Deoxyadenosine	84
	Cytidine	13,100	Deoxycytidine	97
	Guanosine	12,800	Deoxyguanosine	98
	Uridine	15,000	Thymidine	250

In order to study conversion of ribose of CMP-^{14}C (labelled in both base and ribose moieties) to deoxyribose without the results being confused by incorporation of cytosine-^{14}C of CMP-^{14}C into deoxycytidine, the incorporation of label from CMP-^{14}C into deoxyadenosine was studied. It was expected that this incorporation would occur by the reactions:

$$\text{CMP-}^{14}\text{C} \longrightarrow \text{dCMP-}^{14}\text{C} \qquad (1)$$

$$\text{dCMP-}^{14}\text{C} \xrightarrow{\text{venom}} \text{deoxycytidine-}^{14}\text{C} \qquad (2)$$

$$\text{Deoxycytidine-}^{14}\text{C} + \text{adenine} \longrightarrow \text{deoxyadenosine-}^{14}\text{C} + \text{cytosine} \qquad (3)$$

It was assumed that reaction (3) would be catalysed by the transglycosylase previously demonstrated in various Lactobacilli (MacNutt, 1952; Roush and Betz, 1958). Since it appeared unlikely that label from CMP-^{14}C would be incorporated into adenine, the radioactivity of deoxyadenosine might be taken to indicate reduction of the ribose moiety of CMP-^{14}C to deoxyribose derivatives.

Extracts of L. leichmannii which had been treated with Dowex-1-chloride were incubated with 0.1 mM CMP-^{14}C (2 $\mu C/\mu mole$, obtained from Schwartz Bioresearch Inc.), 8 mM ATP, 20 mM magnesium acetate, 0.2 mM NADPH, 4 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (0.02 ml. of 20 times diluted Boehringer suspension of the enzyme per ml. of reaction mixture), 36 mM 2-mercaptoethanol, 36 mM potassium phosphate buffer, pH 7.3, 1 mM adenine and 8×10^{-6} M 5,6-dimethylbenzimidazole cobamide coenzyme (CoB_{12}). After incubation at 37° for 1 hour in the dark the tubes containing reaction mixtures were kept in a boiling

water bath for 2 minutes. After cooling, each reaction mixture (total volume, 0.25 ml.) was mixed first with 0.05 ml. of 0.01 M dCMP and then 0.1 ml. of a solution of freeze-dried Russel Viper venom (10 mg./ml.). The reaction mixtures were allowed to stand at 25° for 16 - 24 hours for reactions (2) and (3) to occur, after which the samples were subjected to electrophoresis on Whatman 3MM paper in 0.025 M borax at 3000 volts for 4 hours. Deoxycytidine moved slowly towards the cathode at a slightly greater speed than deoxyadenosine, and both compounds were widely separated from all other ultraviolet-absorbing material. The identity of deoxyadenosine was established in the first instance by its migration rate in the borax buffer (all ribose derivatives migrate rapidly to the anode) and was confirmed by the ultraviolet spectrum of the eluted material, by its behaviour on two-dimensional paper chromatography with the use of butanol-ammonia in the first dimension and 5% Na_2HPO_4 saturated with isoamyl alcohol in the second dimension and by the fact that it was formed only in the presence of deoxycytidine and ATP and/or adenine.

All constituents of the reaction mixture except NADPH were essential for optimal incorporation of label into deoxyadenosine (Table 2). Since omission of the glucose-6-phosphate dehydrogenase and its substrate caused a considerable drop in deoxyribose labelling, it is clear that glucose-6-phosphate is the ultimate reductant of CMP in this system. Since added NADPH was not required in the presence of glucose-6-phosphate and its dehydrogenase, however, it appears likely that NADP bound so firmly to an enzyme that is not removed by the Dowex-1-chloride, is able to function catalytically in the

electron transport system at maximum enzymic velocity. Omission of adenine did not greatly diminish deoxyadenosine labelling, because under the reaction conditions significant amounts of adenine were formed from ATP. In the absence of CoB_{12} deoxyribose formation from CMP-^{14}C diminished to 1 - 20% of the optimal rate (usually about 5%). The radioactivity of

Table 2

Requirements for the reduction of ribose of CMP-^{14}C by extracts of *L. leichmannii*

Experiment	Omission	Total counts/min. in deoxyadenosine
1	None	3,235
(2.8 mg. of bacterial protein)	CoB_{12}	520
	Mg^{2+}	1,235
	NADPH	3,680
	Glucose-6-phosphate and dehydrogenase	561
	Adenine	2,480
	ATP	357
2	None	1,703
(1.25 mg. of bacterial protein)	CoB_{12}	346
	Mercaptoethanol	136
3	None	3,010
(2.6 mg. of bacterial protein)	None (no incubation at 37° before placing in boiling water bath)	670
4	None	2,745
(3.6 mg. of bacterial protein)	*L. leichmannii* extract	358

the deoxyadenosine formed in the complete system of Expt. 1 represents a synthesis of approximately 1.6 mμmole of deoxyadenosine.

The assay system suffers from the disadvantage that a second relatively long incubation period is required for completion of reaction (3). This is necessary because although the N-transglycosylase is relatively stable at 100° (Roush and Betz, 1958) most of this enzymic activity is lost during the 2 minute period at 100° which precedes the second incubation. Repeated controls have firmly established, however, that no reactions occurring during this second incubation invalidate the assay method. Thus little radioactivity is incorporated into deoxyadenosine when the reaction mixture is transferred to a boiling water bath without incubation at 37° (Expt. 3, Table 2) or when the normal procedure is carried out with a reaction mixture containing no L. leichmannii extract (Expt. 4, Table 2). Furthermore, incorporation of ^{14}C into deoxyadenosine is linearly related to the period of incubation at 37° (up to 1 hour) and to the amount of L. leichmannii protein (up to 4 mg.).

Although elucidation of the function of CoB_{12} in this system requires further investigation it appears unlikely that it is merely keeping an enzyme in a reduced, active state by catalysing reduction of the enzyme by mercaptoethanol as is the case in the stimulation of bacterial pyruvate oxidase by cobamides (Peel, 1962; Rabinowitz, 1960). In contrast to the pyruvate oxidase system, the system for deoxyadenosine-^{14}C formation has just as great a requirement for CoB_{12} under strictly anaerobic conditions as under aerobic. Furthermore, several cobamides and Factor B which

are more active than CoB_{12} in catalysing reduction of pyruvate oxidase are without effect on deoxyadenosine formation.

A more detailed report of this investigation will be published elsewhere.

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