DEOXYCYTIDYLATE FORMATION FROM CYTIDYLATE WITHOUT GLYCOSIDIC CLEAVAGE IN LACTOBACILLUS LEICHMANNII EXTRACTS CONTAINING VITAMIN B_{12} COENZYME

Richard Abrams and S. Duraiswami

Research Department, Montefiore Hospital, and Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pa., 15213

Received December 11, 1964

The observation that deoxyribonucleosides replaced the vitamin B_{12} requirement for growth of several species of Lactobacillus implied that vitamin B_{12} was involved in deoxyribose synthesis in these organisms (Shive et al, 1948; Snell et al, 1948; Wright et al, 1948). A number of investigators have, in fact, found that labeled ribosides were converted to labeled deoxyribotides when L. leichmannii was grown in the presence of vitamin B_{12}, but that the labeled ribose portion was not utilized when these cells were grown with a deoxyriboside in place of the vitamin (Wacker et al, 1959; Floyd and Whitehead, 1960; Manson, 1960). It has recently been pointed out that these tracer experiments constitute no proof for a role for vitamin B_{12} since utilization of labeled riboside stopped immediately upon the addition of a deoxyriboside even in the presence of vitamin B_{12} (Duraiswami and Abrams, 1964).

That vitamin B_{12} does in fact play a role in deoxyribose synthesis in L. leichmannii has recently been demonstrated by Blakley and Barker (1964) with cell free extracts which had been treated with Dowex-1-chloride. In this system as much as 1.67 mμmoles of

deoxyribose were formed from labeled CMP with an absolute requirement for an NADPH generating system, ATP, mercaptoethanol, and 5,6-dimethylbenzimidazole cobamide coenzyme (DMBC). The method of assay did not permit comparison of the pentose/base isotope ratios in precursor and product so that it was not possible to conclude with certainty that reduction of the sugar occurred without prior rupture of the glycoside bond. We have observed repeatedly with cells grown in the presence of vitamin B_{12} and randomly labeled cytidine that the pentose/cytosine ratio in DNA is only half that in RNA, a result compatible with extensive transdeoxyribosylation (MacNutt, 1952) or with cleavage of the cytosine-ribose bond prior to reduction. The results reported here with soluble extracts that have been freed of endogenous deoxyribosides demonstrate that the reduction in L. leichmannii does, in fact, occur without glycosidic cleavage in the presence of ATP, Mg^{++}, dihydrolipoate, and a cobamide.

L. leichmannii ATCC 7830 was grown for 20 hours in 200 ml Difco B_{12} assay medium containing either vitamin B_{12} (1 μg/liter) or deoxycytidine (10 mg/liter). The culture was then diluted with 1800 ml of the same medium and growth continued for 5 hours while gassing with a slow stream of nitrogen. The cells (4 gm wet weight) were centrifuged down, washed with 0.14 M NaCl, and suspended in 12 ml 0.03 M potassium phosphate buffer, pH 7.25. After passage through a French pressure cell, the suspension was centrifuged for 1 hour at 100,000 x g to yield a clear yellow extract containing approximately 15 mg protein/ml. These extracts retained full activity during several months of frozen storage.

When the extract was incubated with CMP-U-C^{14}, labeled dCMP

was formed as shown in Table I. In confirmation of the results of Blakley and Barker (1964), C^{14} appeared in deoxyribose only in the presence of DMBC. However, the extensive loss of C^{14} from the pentose moiety relative to the cytosine portion of the molecule raised the question of whether or not direct reduction without glycosidic cleavage had indeed occurred.

Table I

Instability of Cytosine-Pentose Bond During dCMP Formation from CMP-U-C^{14}

DMBC	Labeled precursors		Labeled dCMP formed		
		Ribose / Cytosine	Cytosine	Deoxyribose	Deoxyribose / Cytosine
μM		C^{14} ratio	mμmoles	mμmoles	C^{14} ratio
15	CMP-U-C^{14}	1.38	4.96	1.32	0.37
0	CMP-U-C^{14}	1.38	2.08	0	0
15	CMP-2-C^{14} + G6P-U-C^{14}	0	5.25	0.03	0

Samples were incubated 30 min. at 37° in 0.25 ml 0.03 M K phosphate buffer, pH 7.25, containing 1.5 mg enzyme protein, 0.008 M ATP, 0.02 M $MgCl_2$, 2 x 10^{-4} M NADPH, 0.004 M glucose 6-phosphate (G6P), 0.5 units G6P dehydrogenase, 0.036 M mercaptoethanol, and 2.2 x 10^{-4} M CMP. Precursor specific activities (cmp per μmole) were: CMP-U-C^{14}, 6.53 x 10^6; CMP-2-C^{14}, 8.35 x 10^6; and G6P-U-C^{14}, 2.94 x 10^6. After incubation, carrier dCMP was added and proteins were removed by acid precipitation. The solutions were hydrolyzed (15 min. at 100° in 0.5 M perchloric acid) and dCMP isolated by chromatography on Dowex-50. Cytosine was liberated by heating for 75 min. at 100° in 70% perchloric acid and purified on Dowex-50. Ribose and deoxyribose specific activities were calculated by difference.

The fact that randomly labeled glucose 6-phosphate did not serve as a precursor of deoxyribose tended to eliminate independent synthesis of the unlabeled deoxyribose. An alternative possibility was deoxyribosyl exchange with an endogenous pool of unlabeled deoxyribosides. To eliminate such a pool, the extract was incubated for 5 hours at 4°

with crystalline DNase (0.5 mg/ml) and then passed through a Sephadex G-25 column to remove low molecular weight components. With this extract, as shown in Table II, dCMP was formed from CMP with no change in the pentose/cytosine C^{14} ratio.

Table II

Stability of the Glycosidic Bond During dCMP Formation with a DNase-Sephadex Treated Extract

Incubation time	Enzyme protein	dCMP formed	Deoxyribose/Cytosine
min	mg	mμmoles	C^{14} ratio
10	1.0	5.01	1.42
20	1.0	7.11	1.30
30	0.35	3.06	1.29
30	0.70	6.21	1.40
CMP-U-C^{14} precursor:			1.38

Incubation was carried out in 0.25 ml 0.03 M K phosphate buffer, pH 7.25, containing 0.008 M ATP, 0.02 M $MgCl_2$, 0.002 M dihydrolipoate, 2.2×10^{-4} M CMP-U-C^{14}, and 7.5×10^{-6} M DMBC.

It is apparent, therefore, that in L. leichmannii, as in E. coli (Reichard and Rutberg, 1960) and mammalian cells (Moore and Hurlbert, 1960; Abrams et al, 1960) deoxyribose is formed by reduction of a nucleotide to a deoxynucleotide.

The optimal requirements for dCMP formation in L. leichmannii extracts are summarized in Table III.

The ATP and Mg^{++} concentrations were not critical and could be varied widely with only secondary effects on the reaction rate. The indicated concentrations of CMP and DMBC were well above saturation levels. Vitamin B_{12} could replace DMBC in this reaction but the resulting activity was only 15% that observed with DMBC. Dihydrolipoate appeared

Table III

Optimal Requirements for dCMP Formation

Omissions	dCMP formed
	mμmoles/10 min/0.5 mg protein
None	5.42
ATP	0.06
DMBC	0.06
Dihydrolipoate	0.07

Incubation was carried out for 10 min in 0.25 ml 0.03 M phosphate buffer, pH 7.25, containing 0.007 M ATP, 0.01 M $MgCl_2$, 2.2×10^{-4} M CMP-U-C^{14}, 0.024 M dihydrolipoate, and 6.3×10^{-6} M DMBC.

to have a low affinity for the enzyme, the saturation level exceeding 0.03 M. This is in marked contrast to the E. coli and mammalian enzymes where saturation occurred at approximately 10^{-3} M. At high dihydrolipoate concentrations, observed activities were in the range of 250 mμmoles/hour/mg of protein, a rate adequate to account for the in vivo rate of DNA synthesis.

It has not been possible to distinguish between extracts of cells grown with vitamin B_{12} and those grown with deoxycytidine. Both were inactive in the absence of DMBC and equally active with optimal levels of added DMBC, suggesting that deoxynucleotides do not repress synthesis of the enzymes involved in their formation.

Acknowledgement - This investigation was supported by Public Health Service Research Grant No. CA-03476 from the National Cancer Institute. Miss Mary C. Stezoski rendered valuable technical assistance.

REFERENCES

Abrams, R., Libenson, L., and Edmonds, M., Biochem. Biophys. Research Communs., 3, 272 (1960).

Blakley, R. L., and Barker, H. A., Biochem. Biophys. Research Communs., 16, 391 (1964).

Duraiswami, S., and Abrams, R., Sixth Int'l. Cong. Biochem., Abstracts, p. 52 (1964).

Floyd, K. W., and Whitehead, R. W., Biochem. Biophys. Research Communs., 3, 220 (1960).

MacNutt, W. S., Biochem. J., 50, 384 (1952).

Manson, L. A., J. Biol. Chem., 235, 2955 (1960).

Moore, E. C., and Hurlbert, R. B., Biochim. et Biophys. Acta, 40, 371 (1960).

Reichard, P., and Rutberg, L., Biochim. et Biophys. Acta, 37, 554 (1960).

Shive, W., Ravel, J. M., and Eakin, R. E., J. Am. Chem. Soc., 70, 2614 (1948).

Snell, E. E., Kitay, E., and MacNutt, W. S., J. Biol. Chem., 175, 473 (1948).

Wacker, A., Kirschfeld, S., and Trager, L., Z. Naturforsch., 14B, 145 (1959).

Wright, L. D., Skeggs, H. R., and Huff, J. W., J. Biol. Chem., 175, 475 (1948).