TRANSFER OF HYDROGEN IN THE COBAMIDE-DEPENDENT RIBONUCLEOTIDE REDUCTASE REACTION*

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Ribonucleotide reductases from Lactobacillus leichmannii and Escherichia coli B have certain notable similarities and differences. Both enzyme systems require a sulfhydryl reductant such as reduced thioredoxin or dihydrolipoate (Laurent et al, 1965; Vitols and Blakley, 1965; Beck et al, 1966); however, the former reduces ribonucleoside triphosphates (Abrams, 1965) and requires DBC coenzyme+ (Blakley and Barker, 1964; Beck and Hardy, 1965; Blakley et al, 1965; Goulian and Beck, 1966a and 1966b) and the latter reduces ribonucleoside diphosphates (Reichard, 1962) and is cobamide-independent (Holmgren et al, 1965). The seeming parallel between the two reactions, and the fact that only one requires DBC coenzyme, raise interesting questions concerning the reaction mechanisms and the role of the coenzyme, especially since other reactions known to require DBC coenzyme are not net reductions.

Larsson (1965) has recently reported that in reducing CDP a partially purified cobamide-independent enzyme system from E. coli B transfers tritium exclusively and nonexchangeably from H_2O-^3H to C-2' of dCDP. The present study, patterned after that of Larsson, demonstrates that tritium is transferred from

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+ Abbreviation: DBC coenzyme, 5,6-dimethylbenzimidazolylcobamide 5'-deoxyadenosyl.

H_2O-^3H to C-2' of dCTP in the cobamide-dependent reduction of CTP by reductase purified from L. leichmannii.

MATERIALS AND METHODS

Experiments were performed with a purified preparation of ribonucleotide reductase from L. leichmannii that is monodisperse on sedimentation analysis and essentially homogeneous under conditions of sedimentation equilibrium according to Yphantis (1964). One mg of enzyme reduces 550-600 mμmoles of CTP per min under standard conditions. These and details of the purification procedure are described elsewhere (Goulian and Beck, 1966b).

Yeast alcohol dehydrogenase (Worthington) was dialyzed against 0.05 M Tris-Cl buffer, pH 7.5, to remove ammonium sulfate. Deoxyribose 5-phosphate aldolase was purified from Lactobacillus plantarum according to Pricer and Horecker (1960). Deoxyribose 5-phosphate was prepared from dAMP according to Lampen (1957). Dihydrolipoate was prepared according to Gunsalus and Razzell (1957) and stored as previously described (Biswas et al, 1965). H_2O-^3H was purchased from New England Nuclear; dl-lipoic acid (disulfide form) from Sigma Chemical; Carbowax 1540 and Haloport F (30-60 mesh) from F & M Scientific; and rhodium catalyst (5% rhodium on alumina) from Baker. DBC coenzyme was a gift of Dr. D. Perlman, Squibb Research Institute.

Ethanol was assayed according to Kaplan and Ciotti (1957); glyceraldehyde 3-phosphate as alkali-labile P according to Beck (1957); and deoxyribose according to Dische (1930). Deoxyribonic acid 5-phosphate was determined as total P (Leloir and Cardini, 1957) in solutions free of diphenylamine-positive material.

CMP and dCMP were separated on a 0.64 cm^2 X 14 cm Dowex-50(H^+) column (Reichard, 1962; Biswas et al, 1965); deoxyribose 5-phosphate, glyceraldehyde 3-phosphate, and deoxyribonic acid 5-phosphate were isolated on 0.64 cm^2 X 14 cm Dowex-2(acetate) columns (Larsson, 1965). Ethanol was separated from H_2O in

100% yields by gas chromatography on an Aerograph 1520 (Wilkins Instruments).*

RESULTS

Transfer of Tritium from H_2O-^3H to dCTP--To determine if tritium is transferred from H_2O-^3H to product during CTP reduction, the following mixture was incubated in the dark at 37° for 1 hr: CTP, 4.2 μmoles; DBC coenzyme, 0.004 μmoles; dihydrolipoate, 30 μmoles; ATP, 8 μmoles; $MgCl_2$, 20 μmoles; Tris-Cl, pH 7.5, 62.5 μmoles; ribonucleotide reductase (hydroxylapatite fraction per Goulian and Beck, 1966b), 64 μg; and H_2O-^3H, 0.37 ml, 0.37 C; in a total volume of 1.6 ml. The incubation mixture contained 3.82 X 10^{10} cpm (specific radioactivity, 2.16 X 10^5 cpm/μg-atom hydrogen).

After incubation the reaction mixture was lyophilized to remove water and other volatile tritiated materials. The residue was lyophilized twice more, 0.4 ml of 15% perchloric acid was added, and the mixture was heated in

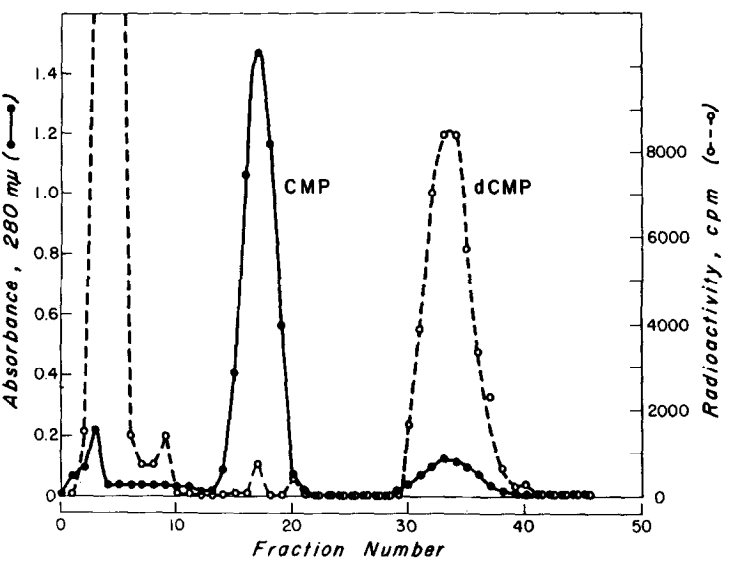

Fig. 1. Dowex-50(H^+) separation of incubation mixture after acid hydrolysis. Elution curves show absorbance at 280 mμ and radioactivity.

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steam for 20 min to convert nucleoside triphosphates to monophosphates. The solution was neutralized with 2.8 N KOH and cleared by centrifugation. The supernatant fraction, containing CMP and dCMP, was lyophilized, and trapped volatile material was shown to be essentially free of tritium. The residue was redissolved in water, adjusted to pH 5.5, separated on Dowex-50(H^+), and assayed for radioactivity (Fig. 1).

The CMP peak contained little tritium; the dCMP peak coincided with a peak of radioactivity. Assays of pooled peaks gave the results in Table 1.

TABLE 1: PRODUCTS OF RIBONUCLEOTIDE REDUCTASE REACTION

Product	Yield	Total radioactivity	Specific radioactivity
	μmoles	cpm	cpm/μmole
CTP (CMP peak)	3.0	700	233
dCTP (dCMP peak)	0.56	39,300	70,400

Calculation based on the specific radioactivity of the original mixture indicates that 0.37 g-atoms of tritium were incorporated per mole of dCTP produced. No tritium was incorporated into dCTP in a parallel experiment in which reductase had been omitted from the incubation mixture.

Isolation of Deoxyribose 5-Phosphate from dCMP--After addition of carrier, dCMP (69.6 μmoles; specific radioactivity, 565 cpm/μmole) was cleaved to deoxyribose 5-phosphate in HCl after the glycosidic bond had been rendered labile by hydrogenation in the presence of rhodium catalyst (Grossman and Greenlees, 1961). The resulting mixture of dihydrocytosine and deoxyribose 5-phosphate was neutralized and separated on Dowex-2(acetate). The isolated deoxyribose 5-phosphate (yield, 40.4 μmoles) had a specific radioactivity of 596 cpm/μmole. Thus, the tritium incorporated initially was associated exclusively with the deoxyribose 5-phosphate moiety of dCTP. The Ba salt of deoxyribose 5-phosphate, prepared according to Pricer and Horecker (1960), had a specific radioactivity of 478 cpm/μmole.

Degradation of Radioactive Deoxyribose 5-Phosphate--After the conversion of Ba salt to Na salt (Pricer and Horecker, 1960), the following mixture was incubated in a 1 cm cuvette at 23° for 50 min: deoxyribose 5-phosphate (Na), 0.5 μmoles; potassium maleate buffer, pH 6.3, 30 μmoles; DPNH, 3 μmoles; deoxyribose 5-phosphate aldolase, 100 units;* and alcohol dehydrogenase, 1 mg, in a volume of 0.65 ml. The decrease in A_{340} indicated formation of 0.5 μmoles of ethanol. Ethanol and water, removed from the reaction mixture by bulb-to-bulb lyophilization, were resolved by gas chromatography.+ The specific radioactivity of isolated ethanol was 418 cpm/μmole. Thus, most of the incorporated tritium was associated with C-1' or C-2' of deoxyribose (Table 2).

TABLE 2: SUMMARY OF THE DEGRADATION OF DEOXYRIBOSE 5-PHOSPHATE FROM RADIOACTIVE DEOXYCYTIDINE TRIPHOSPHATE

	Specific radioactivity
	cpm/μmole
Deoxyribose 5-phosphate, barium salt	478
↓ Ethanol	418
+ Glyceraldehyde 3-phosphate	< 25
Deoxyribonic acid 5-phosphate	425

To remove hydrogens from C-1', 1.5 μmoles of radioactive deoxyribose 5-phosphate (Na) were oxidized for 2 hr at 23° with 50 μmoles of Br_2 in 0.3 ml of water containing 8 μmoles of sodium benzoate (Hudson and Isbell, 1929; Larsson, 1965). Disappearance of diphenylamine-positive material indicated

* A unit of deoxyribose 5-phosphate aldolase is defined as the amount of enzyme causing A_{340} to decrease 0.001 per min in a 0.5 ml mixture containing deoxyribose 5-phosphate, 0.05 μmoles; potassium maleate buffer, pH 6.3, 20 μmoles; DPNH, 0.03 μmoles; and alcohol dehydrogenase, 50 μg.

+ A copper column (6' x 1/4") was packed with Carbowax 1540, 20% (w/w), with Haloport F, 80% (w/w), as solid support. Injection volumes were 100 μl; carrier gas, He (65 psi, 20 ml/min); column temperature, 85-90°; injection temperature, 230°; detector temperature, 250°. Fractions were collected into evacuated containers; gases were dissolved directly in scintillation fluid.

completion of the oxidation. The resulting deoxyribonic acid 5-phosphate, after isolation on Dowex-2(acetate), had a specific radioactivity of 425 cpm/μmole (Table 2). Thus, no tritium had been associated with C-1'.

To ascertain whether tritium was associated with C-3', C-4', and C-5', glyceraldehyde 3-phosphate was isolated on Dowex-2(acetate) from the following mixture after incubation at 23° for 1 hr in a 1 cm cuvette: radioactive deoxyribose 5-phosphate (Na), 2.5 μmoles; potassium maleate buffer, pH 6.3, 500 μmoles; DPNH, 2.8 μmoles; deoxyribose 5-phosphate aldolase, 420 units; and alcohol dehydrogenase, 1.5 mg, in a volume of 3.3 ml. The lyophilized, pooled peak contained 1.3 μmoles of alkali-labile P. Glyceraldehyde 3-phosphate had a specific radioactivity of < 25 cpm/μmole (Table 2); thus, virtually no tritium was associated with C-3', C-4', or C-5'.

DISCUSSION

The results indicate that cobamide-dependent ribonucleotide reductase from L. leichmannii catalyzes a transfer of hydrogen from H_2O to C-2' of dCTP that clearly parallels the hydrogen transfer to C-2' of dCDP catalyzed by cobamide-independent reductase from E. coli B (Larsson, 1965). Evidence that hydrogen atoms exchange freely between H_2O and sulfhydryl groups (Fischer et al, 1953) and that dihydrolipoate is stoichiometrically oxidized during ribonucleoside triphosphate reduction (Vitols and Blakley, 1965) suggest that in both reactions hydrogen is transferred from the reductant sulfhydryl to the 2' pentose carbon. Presumably DBC coenzyme is an intermediate hydrogen transferring agent in the cobamide-dependent reaction; however, it is not clear what agent if any plays this role in the cobamide-independent reaction.

A hydrogen transferring function for DBC coenzyme in this reaction is consonant with the recent demonstration that dioldehydrase catalyzes the transfer of 2 hydrogens per mole of coenzyme from 1,2-propanediol-1-^{3}H to DBC coenzyme and from enzymatically and synthetically prepared ^{3}H-DBC coenzyme to propionaldehyde (Frey and Abeles, 1966). In preliminary experiments, ribonucleotide reductase

from L. leichmannii has transferred radioactivity from synthetic ^{3}H-DBC coenzyme H_2O, but not to dCTP (Beck and Abeles, 1966).

SUMMARY

A purified preparation of cobamide-dependent ribonucleotide reductase from Lactobacillus leichmannii catalyzes a transfer of hydrogen from H_2O to dCTP in the course of CTP reduction. Degradation studies indicate that the incorporated hydrogen is associated exclusively with C-2' of the deoxyribosyl moiety.

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