# TRANSFER OF HYDROGEN FROM COBAMIDE COENZYME TO WATER DURING ENZYMATIC RIBONUCLEOTIDE REDUCTION*

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Recent studies of the reaction mechanism of dioldehydrase have shown that the enzyme catalyzes transfers of hydrogen from C-1 of the substrate (1,2-propanediol-1-$^3$H) to DBCC,+ and from DBCC-5'-$^3$H to C-2 of the product (propionaldehyde) (Frey and Abeles, 1966). In view of these indications that cobamide coenzyme functions as a hydrogen transferring agent in this reaction, it was of interest to consider its role in the cobamide-dependent ribonucleotide reductase reaction (Goulian and Beck, 1966), a net reduction in which tritium is transferred exclusively and nonexchangeably from $H_2O$-$^3$H (or from the freely tritiated -SH groups of the essential reductant, dihydrolipoate) to C-2' of the deoxyribosyl moiety of the product (Gottesman and Beck, 1966). The two reactions are similar in that both involve

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+ Abbreviations: DBCC, 5,6-dimethylbenzimidazolylcobamide 5'-deoxyadenosyl coenzyme; DBCC-5'-$^3$H, synthetically prepared DBCC containing tritium attached to C-5' of the deoxyadenosyl moiety.

displacement of an -OH group by hydrogen. To determine if the mechanism of coenzyme function is similar in the two reactions, experiments were performed to determine the fate of tritium during the enzymatic reduction of CTP in the presence of DBCC-5'-$^{3}$H.

## MATERIALS AND METHODS

The enzyme used was the hydroxylapatite fraction of purified ribonucleotide reductase from Lactobacillus leichmannii (Goulian and Beck, 1966). The enzyme is monodisperse on sedimentation analysis and essentially homogeneous under conditions of sedimentation equilibrium (Yphantis, 1964). The enzyme preparation reduces 550-600 mμmoles of CTP per minute under standard conditions. These and routine assay procedures are described elsewhere (Goulian and Beck, 1966). The synthesis of DBCC-5'-$^{3}$H has been described briefly (Frey and Abeles, 1966).

## RESULTS

The following mixture was incubated in the dark at 37° for 30 min: CTP, 5 μmoles; dihydrolipoate, 60 μmoles; dATP, 0.8 μmoles; DBCC-5'-$^{3}$H, 12 mμmoles (41,500 cpm); magnesium acetate, 32 μmoles; Tris-succinate, pH 7.5, 100 μmoles; and ribonucleotide reductase, 920 μg, in a total volume of 2.0 ml. The reaction was terminated by the addition of 2.0 ml of 20% perchloric acid and the mixture was heated in steam to convert nucleoside triphosphates to monophosphates. The supernatant fraction, containing CMP and dCMP, was adjusted to pH 5.5 and separated on Dowex-50($H^{+}$) (Biswas et al, 1965).

The elution profile, shown in Fig. 1, revealed that in the complete incubation essentially all of the added radioactivity emerged early, appearing in fractions 2-4. None was found in substrate or product. In a control incubation lacking enzyme no more than 10% of the added radioactivity was recovered in the initial fractions. Distillation of the pooled radioactive fractions established that virtually all of the radioactivity (97%) was present in the water of the reaction mixture. It is concluded that tritium from DBCC-5'-$^{3}$H is not transferred to substrate or product as in the diol-dehydrase reaction, but to water.

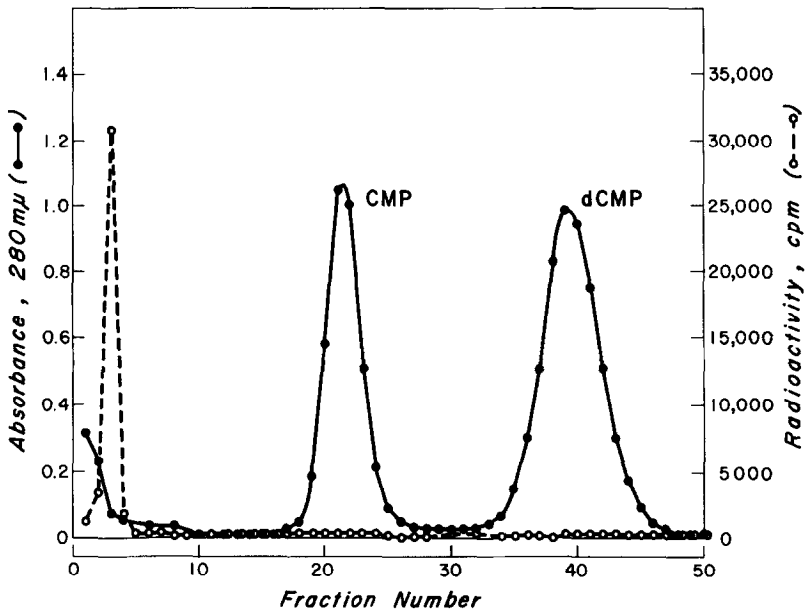


Fig. 1. Dowex-50($H^+$) separation of incubation mixture after acid hydrolysis. Elution curves show absorbance at 280 mμ and radioactivity.

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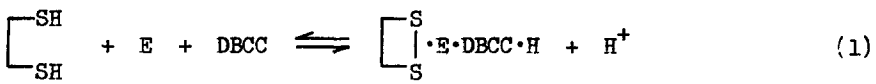
Experiments performed to determine which components of the reaction mixture are required for the transfer of tritium from DBCC-5'-$^3$H to water are summarized in Table 1. These experiments, performed by distillation, recovery, and radioassay of $H_2O$-$^3$H without prior acid treatment, reveal an absolute requirement for substrate, enzyme, and dihydrolipoate. Omission of dATP, an allosteric effector (Beck et al, 1966), significantly decreased CTP reduction but only slightly diminished release of tritium into water. However, the rates of dCTP production shown in Table 1 indicate that at the enzyme concentration used, the molar ratio of product formed to coenzyme added was well above unity, even in the absence of dATP. Under these conditions, coenzyme participation is catalytic and tritium exchange is complete.

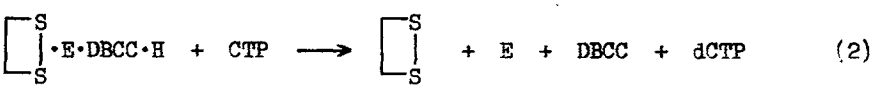
The results differ from those obtained with dioldehydrase in that tritium from synthetic DBCC-5'-$^3$H is not transferred to the product but

is exchanged with the hydrogen of the solvent. The following reaction sequence is postulated for the reduction of ribonucleotides to account for the observed results:




(1)

dihydrolipoate




(2)

lipoate

[E represents enzyme;  ·E·DBCC·H designates an enzyme-bound complex consisting of oxidized lipoate and a reduced form of DBCC.]

If the exchange represented by Eq. 1 is more rapid than the hydrogen transfer in Eq. 2, no tritium should be found in the product. In addition, isotope discrimination would tend to keep tritium from the reaction product (Gottesman and Beck, 1966). If a reaction sequence analogous to that in Eqs. 1 and 2 is written for the reaction catalyzed by dioldehydrase it can be seen that equilibration between the hydrogen of solvent and that of the coenzyme will not occur, since the transferred hydrogen is never in an exchangeable position. Results previously obtained with dioldehydrase (Wagner et al, 1966) have indicated that the enzyme-coenzyme complex could serve as a hydride acceptor in the oxidation of glycolaldehyde to glyoxal. This suggests that a reduced form of the coenzyme occurs as a reaction intermediate.

## SUMMARY

A purified preparation of cobamide-dependent ribonucleotide reductase from Lactobacillus leichmannii catalyzes an exchange of hydrogen between water and cobamide coenzyme in the course of CTP reduction. The exchange is dependent upon the reductase reaction.

TABLE 1: REQUIREMENTS FOR TRANSFER OF TRITIUM FROM DBCC-5'-$^{3}H$ TO $H_2O$

Complete incubation mixture contained: CTP, 0.64 μmoles; dihydrolipoate, 4.8 μmoles; dATP (ATP-free), 64 mμmoles; DBCC-5'-$^{3}H$, 1.5 mμmole (13,600 cpm), magnesium acetate, 2.6 μmoles; Tris-succinate buffer, pH 7.5, 8 μmoles; and enzyme, 50 μg, in a volume of 0.16 ml. Reactions were incubated for 20 min at 37°. Production of dCTP was assayed in parallel incubations containing CTP-2-$^{14}C$ and unlabeled DBCC.

| Incubation mixture | $^{3}H$ in water | dCTP formed |
|---|---|---|
| | % of total | mμmoles |
| Complete system | 97.3 | 281 |
| minus CTP | 0.9 | 0 |
| minus enzyme | 0.9 | 0 |
| minus dihydrolipoate | 2.7 | 0 |
| minus dATP | 89.0 | 20 |
| with less enzyme (5 μg) | 94.6 | 29 |
| with lipoate in place of dihydrolipoate | 0.5 | 0 |

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