Studies on the Mechanism of Enzyme-Catalyzed Oxidation Reduction Reactions.* V. An Exchange Reaction Catalyzed by Liver Alcohol Dehydrogenase

V. P. Fernandez, H. R. Mahler, and V. J. Shiner, Jr.

From the Department of Chemistry,† Indiana University, Bloomington, Indiana

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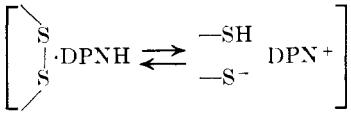
In order to determine whether a stereospecific transfer of hydrogen from the reduced coenzyme to liver alcohol dehydrogenase occurs, the pure enzyme was incubated with reduced diphosphopyridine nucleotides containing tritium in the 4-α- or 4-β-position of the dihydropyridine ring. After denaturation and lyophilization a significant proportion of the radioactivity added in the former, but not the latter, case appeared in the volatile fraction, and was shown to be contained in ethanol introduced originally in a form tightly bound to enzyme. This hydrogen transfer–exchange was enhanced by the addition of ethanol to the reaction mixture. In the case of yeast alcohol dehydrogenase a comparable reaction was absolutely dependent on the presence of alcohol.

Attempts to elucidate the mechanism of action of liver alcohol dehydrogenase (LADH) with alcohol or acetaldehyde as substrates in this laboratory (Shiner *et al.*, 1960; Mahler *et al.*, 1962) as well as elsewhere (Theorell and Chance, 1951; Theorell, 1959; Theorell and McKee, 1961, and references therein; Shifrin and Kaplan, 1960) have indicated that the most likely path involves the formation of a binary complex between enzyme and DPN^+ or DPNH as an obligatory first step. It has also been suggested that there is a more or less complete transfer of the active hydrogen from DPNH to the enzyme on binding and that this binding between DPNH and enzyme occurs perhaps at a disulfide linkage of the latter, with the resulting thiol proton blocked toward exchange in the native enzyme (Baker, 1960).



(1)

Indeed, in this earlier work all attempts to prove the occurrence of this reaction with the native enzyme protein were unsuccessful but some success attended its demonstration by sulfhydryl titration of an enzyme denatured by heat in the presence of DPNH. The interpretation of these results, however, was not without ambiguity, and a more direct proof appeared desirable.

The present studies were therefore undertaken with the view of showing that the mechanism shown in equation (1) is operative. With tritium used in place of the active hydrogen in DPNH, a transfer of the label to the disulfide linkage should give rise to a sulfhydryl group which might, especially after denaturation, readily exchange protons with the aqueous solvent medium. The resulting water after equilibration should then be enriched with tritium with measurable radioactivity.

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Experimental

Materials.—Diphosphopyridine nucleotide, oxidized form (DPN^+), 97.5% pure, was obtained commercially from Sigma Chemical Co., St. Louis, Mo.

Enzymes.—Twice-crystallized horse liver alcohol dehydrogenase, as either a dry powder or a suspension in phosphate buffer at dry ice temperatures, was supplied by Worthington Biochemical Corporation, Freehold, N. J. Before use this preparation was recrystallized once more according to the procedure of Bonnichsen and Brink (1958) and suspended in glycylglycine–sodium hydroxide buffer, *p*H 8.5. Specific activities from 70–97% according to the assay of Bonnichsen and Brink (1958) were obtained. This enzyme contains alcohol, relatively tightly held by the protein. When alcohol-free samples of the enzyme were needed, the enzyme solution after recrystallization was first dialyzed against 0.01 M phosphate buffer, *p*H 7.0, for a period of 24 hours with several changes of the buffer solution and then lyophilized and the dry enzyme resuspended in glycylglycine buffer. Activity of the lyophilized enzyme was always found to be lower than that of the starting material (50–70%). Previous unpublished studies by R. H. Baker, Jr., have also indicated that the various kinetic parameters determined for this alcohol-free enzyme do not correspond to those obtained with the fully active, alcohol-containing enzyme, and that, furthermore, the apparent mechanisms of action of the two types of enzyme preparation are not identical. The absorption coefficient used for the determination of this protein was $a_{280}^{mg/ml} = 0.455$, and the molecular weight was assumed to be 73,000, with two equivalent active sites/molecule (Bonnichsen and Brink, 1958).

Yeast Alcohol Dehydrogenase.—Yeast alco-

hol dehydrogenase, prepared by Boehringer, Stuttgart, Germany, and supplied by California Corporation for Biochemical Research, Los Angeles, in saturated ammonium sulfate was recrystallized once (Racker, 1958); two different preparations had specific activities of 18 and 57 units/mg of protein respectively. For this protein, concentration was determined by the method described by Warburg and Christian (1941) and modified by Layne (1958); the molecular weight was assumed to be 150,000, with four equivalent active sites/molecule (Hayes and Velick, 1954).

GLUCOSE DEHYDROGENASE.—Glucose dehydrogenase was prepared in the laboratory from fresh beef liver (Brink, 1953).

PREPARATION OF TRITIUM-LABELED DPN$^+$.—The method employed for this preparation was essentially that of Jarabak and Talalay (1960) in which the basic cyanide complex of DPN$^+$ was equilibrated with tritiated water prior to decomposition. Since the DPN$^+$-4-T concentration in the effluent liquid upon chromatography was very low, it was concentrated to a smaller volume (100 ml) either by lyophilization or by flash evaporation before precipitation with acetone. Starting with 714 mg of 97.5% DPN$^+$, a yield of 408 mg of dry product was obtained which upon assay with ethanol and alcohol dehydrogenase proved to be 76% pure by weight. Specific activity of the product was 3879 ± 26 cpm per μmole on the basis of a molecular weight of 750.

4-β[1]-TRITIO-DPNH (DPNH-β-T).—DPN$^+$-4-T was reduced enzymatically with alcohol dehydrogenase as described by Rafter and Colowick (1958).

4-α[1]-TRITIO-DPNH (DPNH-α-T).—The procedure used the unpublished method of Mahler and Suzue for the preparation of DPNH-α-D by means of reaction (2) catalyzed by the enzyme glucose dehydrogenase.

$$\text{D-glucose} + \text{DPN}^+\text{-4-T (or -D)} \rightleftharpoons \text{D-gluconolactone} + \text{DPNH-}\alpha\text{-T (or -D)} \quad (2)$$

A typical preparation was as follows: DPN$^+$-4-T (20 mg) and D-glucose (180 mg) were dissolved in 1 ml of 0.5 M tris(hydroxymethyl)aminomethane (Tris). One ml of 0.5 M Tris containing some 3 units of glucose dehydrogenase was added and the mixture allowed to stand at room temperature for half an hour. When the reduction was complete as determined spectrophotometrically at 340 mμ the product was separated as in the case of DPNH-β-T. Yields of the product were almost quantitative.

It was found that neither glutamic acid dehydrogenase nor glyceraldehyde phosphate dehydrogenase catalyzing reactions (3) and (4) was suitable for preparing the α-isomer, since the reaction did not go to completion in (3) and isolation posed a problem with (4).

$$\text{L-glutamate} + \text{DPN}^+\text{-4-T} + \text{H}_2\text{O} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{DPNH-}\alpha\text{-T} \quad (3)$$

[1] The stereospecificity and nomenclature of these compounds have been reviewed by Vennesland (1958).

$$\text{D-glyceraldehyde-3-phosphate} + \text{DPN}^+\text{-4-T} + \text{H}_2\text{O} \xrightarrow[\text{HAsO}_4^{=}]{} \text{D-glycerate-3-phosphate} + \text{DPNH-}\alpha\text{-T} + \text{H}^+ \quad (4)$$

Counting Procedure.—The radioactivity of the samples was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 314X. DPN$^+$-4-T of known weight was dissolved in 1 ml of hydroxide of Hyamine 10-X[2] and 20 ml of scintillator solution in a counting bottle. Solutions of the aqueous lyophilizate were made up as follows for counting: 0.3 ml of lyophilizate, 5 ml of absolute ethanol, and 15 ml of scintillator solution. The scintillator solution had the following composition: 4 g of 2,5-diphenyloxazole, 100 mg of 1,4,-*bis*-2-(5-phenyloxazolyl)-benzene in one liter of toluene. It was found that the quenching factor with 1 ml of hydroxide of Hyamine was almost identical to that with 0.3 ml of water.

Equilibration Studies.—DPNH and alcohol dehydrogenase of known concentrations were incubated at room temperature in glycylglycine buffer *p*H 8.5 for 5 minutes. Denaturation of the enzyme was effected either by heating the mixture for 5 minutes in boiling water, taking care to collect all water with an efficient reflux condenser, or by the addition of 1% sodium lauryl sulfate. The mixture was then lyophilized and the radioactivity of the lyophilizate counted. Both methods of denaturation gave identical results.

RESULTS AND DISCUSSION

Solutions of liver alcohol dehydrogenase in glycylglycine buffer, *p*H 8.5, were equilibrated with DPNH-α-T and DPNH-β-T, respectively, the enzyme denatured, and the mixture lyophilized. The lyophilizate was collected and its radioactivity counted. The results of representative experiments are summarized in Table I. In general they were the results predicted on the basis of the hypothesis advanced in the introduction: exchange occurs; it requires the enzyme protein (experiment 1 *vs.* 7 or 8) in native conformation (experiment 1 *vs.* 5) and is stereospecific for the coenzyme (experiment 1 *vs.* 6); and the exchangable protons are made more available after denaturation (experiment 1 *vs.* 3). Nevertheless the exchange phenomenon demonstrated here must have an explanation different from that postulated earlier. In experiments 1 and 3, in which the enzyme recrystallized from ethanol was used, the lyophilizate showed high radioactivity. However, in experiment 2, where the enzyme contained a smaller quantity of ethanol, the activity of the lyophilizate was considerably lower. Unfortunately it has not been possible to perform equilibration studies with liver alcohol dehydrogenase that was completely free of ethanol, since all attempts to rid the enzyme of the substrate by prolonged dialysis followed by lyophilization

[2] Trade name of Rohm and Haas; 1 M solution of *p*-(diisobutyl-cresoxyethoxyethyl)-dimethylbenzyl ammonium hydroxide in methanol.

TABLE I

EQUILIBRATION STUDIES WITH LIVER ALCOHOL DEHYDROGENASE (LADH)

Exper. No.	Reaction	Concentrations: Enzyme × 10^4 M	Concentrations: DPNH × 10^3 M	Total Volume of Reactants (ml)	Cpm/0.3 ml Lyophilizate
1	LADH + DPNH-α-T[a]	3.29	1.30	1	1541
		4.11	1.43	1	1460
2	LADH + DPNH-α-T[a,b]	2.70	1.35	2	252
		5.60	2.60	1	292
3	LADH + DPNH-α-T[c]	3.29	1.49	1	942
4	LADH + DPNH-α-T + 0.17 mmole ethanol (equilibrated for 1 hr.)	Catalytic amount (~0.1)	1.06	1.15	1024
5	LADH[d] + DPNH-α-T[a]	5.48	1.87	1	24
6	LADH + DPNH-β-T[a]	4.11	1.59	1	177
7	Bovine serum albumin + DPNH-α-T[a]	2.92	1.34	1	21
8	DPNH-α-T + buffer[a]		1.94	1	25

[a] Heated in boiling water after 5 minutes of equilibration. [b] Enzyme was freed of most of the ethanol by dialysis. [c] Enzyme was not denatured after equilibration. [d] Enzyme denatured prior to equilibration.

failed. It was therefore decided to analyze the lyophilizate for ethanol. Since this was present in rather small quantities, separation by processes such as distillation could not be employed. The method of Brink and co-workers (1954) was used for determining the ethanol content in the lyophilizate. In order to remove the ethanol it was treated with an excess of DPN^+ and yeast alcohol dehydrogenase. It has been shown by Loewus *et al.* (1953) that the reaction catalyzed by yeast alcohol dehydrogenase is stereospecific for ethanol. Therefore, if the radioactivity is in the originally enzyme-bound alcohol, it should have been introduced by liver alcohol dehydrogenase, and since yeast alcohol dehydrogenase has the same stereospecificity, the radioactivity in the ethanol should be removed by the DPN^+–yeast alcohol dehydrogenase treatment. Accordingly the lyophilizate obtained on treatment of DPNH-α-T with liver alcohol dehydrogenase (henceforth called lyophilizate I) was made 0.5 M in Tris, DPN^+ and yeast alcohol dehydrogenase were added at intervals, and the mixture was then lyophilized. The radioactivity of this lyophilizate (lyophilizate II) was again counted. Results obtained (experiment A, Table II) indicate that most of the radioactivity was in the ethanol. Since one DPN^+ treatment did not completely remove all the radioactivity, lyophilizate II from a separate experiment was subjected to an additional DPN^+–yeast alcohol dehydrogenase treatment. It was found (experiment B, Table II) that the second treatment succeeded in removing tritium from the lyophilizate almost completely.

Yeast alcohol dehydrogenase can be crystallized free of ethanol by the method of Racker (1958).

TABLE II

REMOVAL OF LABEL, ORIGINALLY INTRODUCED WITH LIVER ALCOHOL DEHYDROGENASE (LADH), BY YEAST ALCOHOL DEHYDROGENASE (YADH)–DPN TREATMENT

		Cpm/0.3 ml	Ethanol Content
Exper. A (DPNH-α-T, 8.1 × 10^{-4} M; LADH, 1.8 × 10^{-4} M)	lyophilizate I	710	116 μg/ml
	lyophilizate II	46	5 μg/ml
Exper. B (DPNH-α-T, 1.27 × 10^{-3} M; LADH, 2.5 × 10^{-4} M)	lyophilizate I	463	
	lyophilizate II	48	
	lyophilizate III	28	

When this enzyme was subjected to similar equilibration studies with the two isomers of DPNH-T it was found (Table III) that no activity resulted in the lyophilizate. However, when a trace amount of ethanol was introduced in the equilibrium mixture containing DPNH-α-T the lyophilizate obtained showed activity, indicating that in at least this case there is definite evidence for an obligatory dependence of the exchange phenomenon on the presence of ethanol.

TABLE III

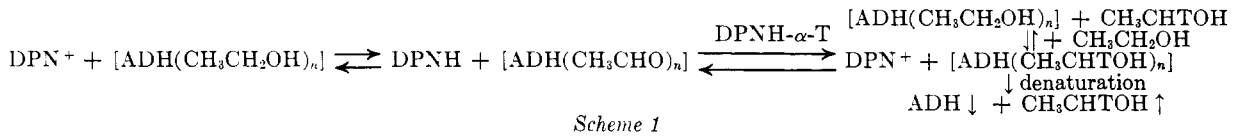
EQUILIBRATION STUDIES WITH YEAST ALCOHOL DEHYDROGENASE (YADH)

Exper. No.	Reaction	Concentrations: Enzyme × 10^4 M	Concentrations: DPNH × 10^3 M	Total Volume of Reactants (ml)	Cpm/0.3 ml Lyophilizate
1	YADH + DPNH-α-T[a]	1.49	1.07	1	38
2	YADH + DPNH-α-T[b]	0.74	1.45	1.4	72
3	YADH + DPNH-α-T	1.49	1.16	1	41
4	YADH (denatured) + DPNH-α-T	1.49	1.19	1	36
5	YADH + DPNH-β-T[a]	1.49	1.82	1	41
6	YADH + (trace ethanol[c]) + DPNH-α-T[a]	1.49	1.78	0.8	1269

[a] Denatured by heating in boiling water for 5 minutes. [b] Denatured by the addition of 1% sodium lauryl sulfate. [c] Approx. 10^{-2} M.

Experiments 4 and 6 in Tables I and III, respectively, show that tritium from DPNH-α-T can also be transferred with use of catalytic amounts of liver alcohol dehydrogenase or yeast alcohol dehydrogenase with small amounts of ethanol present in the equilibration mixture. A plausible mechanism for all the observed phenomena is shown in Scheme 1.

It is possible that the DPNH-α-T contains a minute amount of DPN^+ as postulated, though we have been unable to detect the latter species spectrophotometrically using liver alcohol dehydro-



Scheme 1

genase and ethanol. Samples of DPNH-α-T that were pretreated with sodium hydrosulfite in order to insure complete reduction prior to equilibration gave results similar to those obtained with the untreated DPNH-α-T. It is, however, conceivable that just a few molecules of unreacted DPN^+ are sufficient to produce acetaldehyde, which in turn could react with DPNH-α-T present in relatively large amounts to produce tritium-rich ethanol and regenerating DPN^+, which could again react with the unlabeled ethanol to continue the process. An intriguing but less plausible alternative would postulate a direct, enzyme-catalyzed exchange of hydrogens between DPNH and CH_3CH_2OH, *independent* of the presence of DPN and CH_3CHO. In this context the recent demonstration of catalysis by liver alcohol dehydrogenase of dismutation (Abeles and Lee, 1960) and isomerization reactions (Van Eys, 1961) may be pertinent. It must also be emphasized that the reaction described here is an exchange not with free but with enzyme-bound, relatively tightly held ethanol, present in all preparations of liver alcohol dehydrogenase crystallized by the standard procedure and not removed by such treatments as dissolving the enzyme in buffer or treating it with Sephadex. As can be seen from the data of Table II, this alcohol is present to the extent of 10–20 molecules per molecule of enzyme protein.

The exchange reaction described here, *i.e.*, the stereospecific exchange of label between DPNH-α-T and *alcohol*, *i.e.*, the reduced substrate, in the presence of liver alcohol dehydrogenase or yeast alcohol dehydrogenase, would seem to be a particularly simple and convenient diagnostic for the determination of stereospecificity of pyridine-nucleotide–linked enzymes. All that is required is enzyme, substrate, and samples of DPNH-α- and β-T.

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