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INHIBITOR, ISOTOPIC AND KINETIC STUDIES ON HYDROGEN DEHYDROGENASE

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SUMMARY

The oxidized form of hydrogen dehydrogenase of *Hydrogenomonas ruhlandii* was inhibited by BAL, cyanide, dithionite, sulfide, sulfite and thioglycolate. Reduction of the dehydrogenase with DPNH or H_2 resulted in a diminished effect of the inhibitors, with the exception of *p*-chloromercuribenzoate which inhibited only the reduced enzyme. Information from kinetic analysis and inhibitor studies indicates that the enzyme is reduced with H_2 to give a proton and that this is followed by the oxidation of the reduced enzyme with DPN to give DPNH. The enzyme catalyzes an exchange between H_2 and HTO and also between HTO and DPNH. Analysis of DPN enzymically reduced with T_2 shows that T is in the β-position of DPNH. The enzyme action has a lag phase which can be eliminated by preincubation with H_2 or DPNH.

INTRODUCTION

The mechanism of action of hydrogen activating enzymes has been investigated from the viewpoint of exchange reactions [1,2], the metal component[3,4] and a possible flavin component[5-7]. The hydrogenase of *Desulfovibrio desulfuricans* has been extensively investigated[2,4,8] and RIKLIS AND RITTENBERG[4] have obtained a highly purified preparation using the exchange reaction between deuterium oxide and hydrogen as the assay system. The hydrogenase of *D. desulfuricans* can also reduce benzyl viologen, methylene blue and liberate hydrogen from reduced methyl viologen[4,8]. Spectrographic analysis of this protein showed iron to be the major metal component. The exchange reaction indicated that the hydrogenase reaction can occur as a heterolytic process resulting in the formation of an enzyme hydride and a proton[9].

Hydrogen dehydrogenase of *Hydrogenomonas ruhlandii* provides a more amenable system for the study of the activation of hydrogen and in this report attention has been focussed on inhibitors which can react with disulfide groups, the use of isotopes to evaluate the exchange reactions and a kinetic analysis of the hydrogen dehydrogenase reaction.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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MATERIALS AND METHODS

The following materials were commercial preparations: pig-heart malic dehydrogenase (C. F. Boehringer and Sohne, Mannheim, Germany), liver-glutamic dehydrogenase (Sigma Chemical Company), DPN analogs (Pabst Laboratories).

Hydrogen dehydrogenase used was enzyme B and assayed as described in the accompanying paper[10]. Kinetic measurements were made using 0.1 M Tris–HCl buffer (pH 7.8) saturated with H_2 at 25°. The solubility of H_2 in Tris buffer is assumed to be the same as in water. The technique for the measurement of reduced analogs of DPN is taken from the Pabst Laboratories Circular OR-18. Samples of tritiated material were pipetted onto planchettes and then dried down. Each sample was taken up in 0.2 ml of water and evaporated to dryness 5 times. Radioactivity was then measured using a windowless gas flow counter.

RESULTS

Inhibitors

Table I shows the effect of inhibitors on hydrogen dehydrogenase. The inhibitors listed are reagents which split disulfide bonds[11] and also can, in the case of cyanide and sulfide react with a metal[12]. The reaction of cyanide and sulfide with the oxidized enzyme is a slow reaction and the maximum inhibition is attained only after a 5-min incubation period whereas sulfite reacts instantaneously with the enzyme and

TABLE I

EFFECT OF INHIBITORS ON HYDROGEN DEHYDROGENASE ACTIVITY

Preincubated solutions contained hydrogen dehydrogenase (sufficient enzyme to give an absorbancy change at 340 mμ of 0.2/min in the final volume), and 20 μmoles Tris–HCl buffer (pH 7.8). For DPNH preincubation, the solution contained enzyme, 20 μmoles Tris-succinate (pH 6.9) and 0.06 μmole DPNH. In hydrogen preincubation experiments, hydrogen was passed through the solution. Preincubation was 5 min at 25°, volume of solution 0.2 ml and concentration of inhibitors as indicated. Sephadex treatment, at the end of preincubation, 0.3 ml of solution treated with 0.4 ml of Sephadex G-25 suspension (25% (v/v) in 0.1 M Tris HCl (pH 7.8)) for 5 min, then centrifuged and supernatant assayed. The reaction was started by adding 2.8 ml of solution containing 280 μmoles Tris–HCl (pH 7.8), 2 μmoles DPN and saturated with hydrogen, to the preincubation mixtures.

Additions to preincubation mixture	*Inhibition (%)*	*Additions to preincubation mixture*	*Inhibition (%)*
H_2	0	$5 \cdot 10^{-4}$ M Na_2S (pH 7.8*)	52
DPNH	0	10^{-3} M Na_2SO_3 (pH 7.8)	45
10^{-3} M KCN (pH 7.8)	46	10^{-3} M Na_2SO_3 (pH 7.8) + H_2	0
10^{-3} M KCN (pH 7.8) + H_2	7	$5 \cdot 10^{-3}$ M $Na_2S_2O_4$	68
10^{-3} M KCN (pH 6.9)	97	$5 \cdot 10^{-3}$ M $Na_2S_2O_4$ + H_2	37
10^{-3} M KCN (pH 6.9) + DPNH	46	$3 \cdot 10^{-3}$ M BAL	82
10^{-3} M KCN (pH 7.8) + Sephadex	44	$3 \cdot 10^{-3}$ M BAL + H_2	56
$5 \cdot 10^{-4}$ M Na_2S (pH 7.8)	100	$5 \cdot 10^{-4}$ M Thioglycollate	43
$5 \cdot 10^{-4}$ M Na_2S (pH 7.8) + H_2	46	$5 \cdot 10^{-4}$ M Thioglycollate + H_2	29
$5 \cdot 10^{-4}$ M Na_2S (pH 6.9)	100	$7 \cdot 10^{-5}$ M PCMB	0
$5 \cdot 10^{-4}$ M Na_2S (pH 6.9) + DPNH	75	$7 \cdot 10^{-5}$ M PCMB + H_2	53
$5 \cdot 10^{-4}$ M Na_2S (pH 7.8) + Sephadex	100		

* 10^{-3} M GSSG present in reaction mixture.

the reaction is complete in 30 sec. Attempts to reverse the inhibitions of Na_2S and KCN by (a) dilution thirty-fold with Tris buffer (b) removal of inhibitor by Sephadex G-25 gel under conditions which effectively remove sulfide (Table I) (c) dialysis against 100 volumes of 0.05 M potassium phosphate (pH 7.8), 0.001 M EDTA 0.0007 M $MnCl_2$ for 24 h (d) treatment with 0.001 M $ZnSO_4$ (e) addition of 0.03 M potassium salt of EDTA, were not effective. The only condition which partially reversed the inhibition of sulfide was the dilution of the preincubation mixture with a solution of GSSG (Table I). A DPN-sulfide enzyme complex has been reported as the mechanism of inhibition of lactic dehydrogenase[13]; but in the case of hydrogen dehydrogenase DPN had no effect on the inhibition of the enzyme by $2 \cdot 10^{-4}$ M Na_2S. Further, DPN was found to have no effect on any of the inhibitors except in those known cases where DPN reacts directly with the inhibitor.

The reagents were all more effective when the inhibitor was preincubated with the enzyme and the reaction started by adding H_2 and DPN than when the enzyme was first reduced by H_2 or DPNH and the inhibitor then added. The extent to which the pretreatment affected the inhibition was dependent on the inhibitor (Table I). Variable results for cyanide inhibition have been reported for hydrogenases from anaerobic bacteria[1,3,8,9,14]. The author has found that it is essential to standardize conditions for all inhibitor studies and that special care be given to the oxidized - reduced state of the enzyme, if variable results are to be eliminated.

It was possible to carry out a reduction of hydrogen dehydrogenase with a small amount of H_2 (0.3 μmole) to give an enzyme which is inhibited 44% by $5 \cdot 10^{-4}$ M Na_2S. When the enzyme was then oxidized with 3 μmoles of DPN and the nucleotides removed with Sephadex G-25 gel, an enzyme was obtained which is completely inhibited by sulfide.

PCMB inhibition varies in the reverse manner to that of the other inhibitors, the reduced enzyme being more sensitive to PCMB (Table I). A similar effect was noticed with glutathione reductase which becomes sensitive to sulfhydryl reagents when reduced with TPNH or DPNH[15]. Another inhibitor studied was 10^{-3} M arsenite which inhibited the enzyme 12% whether the enzyme was oxidized or reduced. The effects of varying the concentration of NaCl, urea and Tris-HCl buffer (pH 7.8) were tried in arsenite inhibition experiments using enzymes A and B[10], and enzyme B prepared in the absence of manganese. In no case was the inhibitory effect of arsenite increased. Oxygen was found not to inhibit the enzyme and no precautions were found necessary to ensure the absence of oxygen during the purification procedure[10].

As sulfide may form a stable compound with the enzyme, the enzyme (100 μg) was incubated with $Na_2{}^{35}S$ (0.1 μmole, $2 \cdot 10^6$ counts/min) for 20 min at room temperature. The sulfide and enzyme were separated by the addition of sufficient saturated $(NH_4)_2SO_4$ solution to bring the saturation to 60%. The precipitated protein was washed twice with 60% saturated $(NH_4)_2SO_4$ and its radioactivity then measured. No radioactivity was associated with the protein.

Kinetics of inhibited enzyme

The effects of the inhibitors cyanide, sulfide and sulfite were investigated by measuring the rates of reaction in the presence of a constant amount of enzyme, inhibitor and excess substrate, whilst varying the concentration of the other sub-

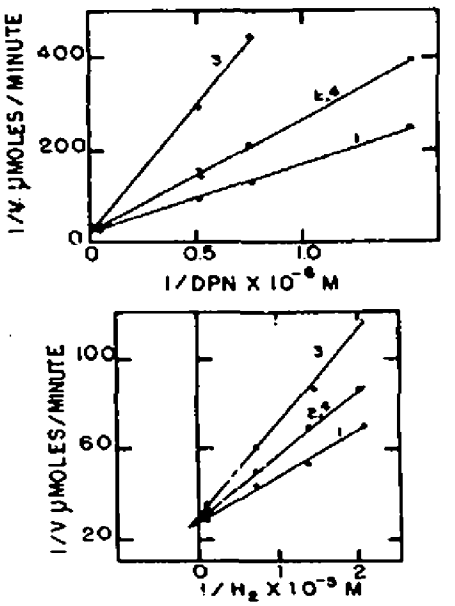

Fig. 1. Effect of cyanide, sulfide and sulfite on hydrogen dehydrogenase. 0.04 unit of oxidized enzyme preincubated as in Table I with 10^{-6} M KCN (Curve 2), 10^{-3} M Na_2SO_3 (Curve 3) and $2.5 \cdot 10^{-6}$ M Na_2S (Curve 1). Reaction started by adding varying amounts of DPN and H_2 saturated buffer.

strate. The Lineweaver–Burk plot was used to analyze the results (Fig. 1). All three inhibitors were competitive with DPN and partially non-competitive with H_2.

Kinetics of reduction of DPN by hydrogen

A lag phase was observed when the enzyme was assayed by adding DPN and a H_2 saturated buffer to the enzyme. No lag phase was found when the enzyme was preincubated with H_2 or DPNH (Fig. 2). The lag phase could also be removed by increasing the concentration of the enzyme (Fig. 2). Other reagents such as GSH, cysteine, ascorbate and ferrous ammonium sulfate were ineffective in removing the lag phase. It was possible to increase the lag phase 3-fold by preincubating the enzyme with 0.1 M NaCl. This effect was not reversed by the addition of H_2 or DPNH.

Plots were made according to the method of LINEWEAVER–BURK on the effect of DPN concentration on its rate of reduction by H_2 and the results shown in Fig. 3 were obtained at different concentrations of H_2. Such kinetics are consistent with the mechanism for a two substrate system in which the enzyme exists in two different states (oxidized and reduced) and the substrate can give one of the products when reacting with a particular form of the enzyme[16]. Eqns. 1 and 2 represent such a mechanism. WINFIELD[17] has proposed Eqn. 1 for the mechanism of hydrogenase.

$$E + H_2 \rightleftarrows EH_2 \rightleftarrows EH^- + H^+ \quad (1)$$

$$EH^- + DPN^+ \rightleftarrows EH \cdot DPN \rightleftarrows E + DPNH \quad (2)$$

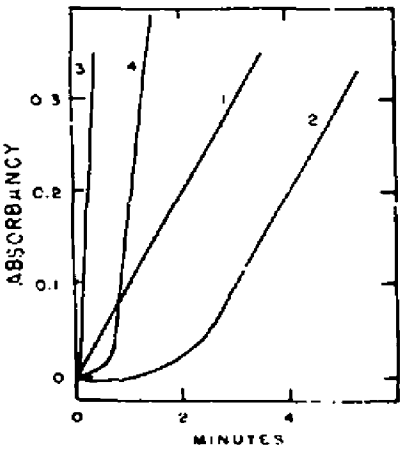

Fig. 2. Effect of enzyme concentration, DPNH and H_2 on lag phase of dehydrogenase reaction. For Curve 1, 0.05 unit of enzyme preincubated with either DPNH or H_2 as described in Table I. For Curve 2, the same as Curve 1 except no DPNH or H_2 added to preincubation mixture. For Curve 3, 0.25 unit of enzyme and Curve 4, 0.15 unit of enzyme were used.

taking as his model catalytic hydrogenation and the results of KRASNA AND RITTENBERG[9]. The rate equation for this mechanism is Eqn. 3

$$V_{max}/v = 1 + K_{H_2}/H_2 + K_{DPN}/DPN \qquad (3)$$

where v is initial velocity, V_{max} maximum velocity, K_{H_2}, K_{DPN} constants and H_2, DPN molar concentration of H_2 and DPN. When the LINEWEAVER-BURK plot is made with equimolar concentrations of substrates which are varied simultaneously,

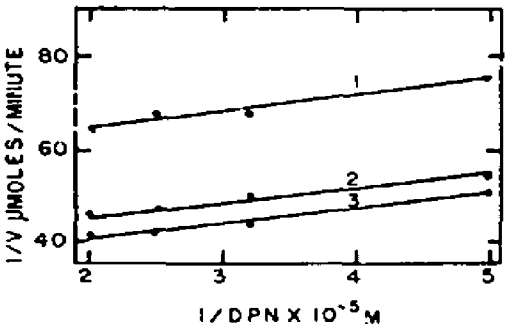

Fig. 3. Effect of DPN and H_2 concentration on the rate of hydrogen dehydrogenase reaction. For Curve 1, 1.6 ml of H_2 saturated buffer; Curve 2, 2.2 ml of H_2 saturated buffer; Curve 3, 2.8 ml of H_2 saturated buffer were used. Reaction started by adding 0.03 unit of enzyme to assay mixture.

Eqn. 3 reduces to a linear relation between the reciprocals of initial velocity and substrate concentration (Fig. 4). Other types of mechanism for two substrates would give a non-linear plot[10].

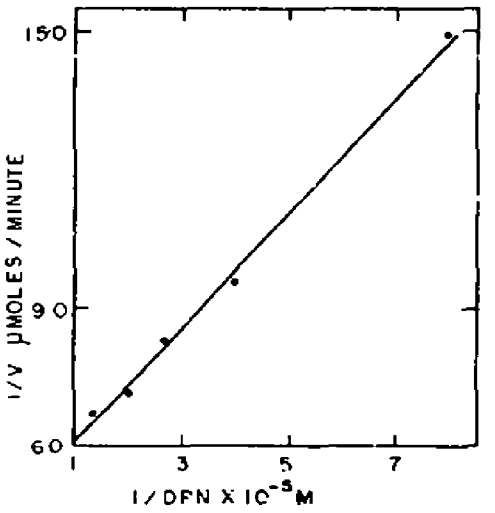

Fig. 4. Effect of equimolar concentrations of DPN and H_2 on the rate of hydrogen dehydrogenase. H_2 saturated buffer contained $7.2 \cdot 10^{-6}$ M DPN at 25°.

Reduction of DPN analogs by hydrogen

Table II gives the relative rates of reduction of DPN analogs. There is a high degree of specificity of reaction as the only analog reduced was deamino-DPN.

Exchange reactions with tritiated water

When the dehydrogenase was incubated with tritiated water and shaken under H_2–He (20 : 80) for 30 min and a sample of gas then withdrawn through a $CaCl_2$

TABLE II

REACTION OF ANALOGS OF DPN WITH HYDROGEN DEHYDROGENASE

Enzyme assayed under standard conditions[10] using $2 \cdot 10^{-4}$ M concentrations of nucleotides.

Nucleotide	*Initial rate (%)*
DPN	100
3-Acetylpyridine-DPN	0
3-Acetylpyridine-deamino-DPN	0
Deamino-DPN	9.3
3-Pyridinealdehyde-DPN	0
3-Pyridinealdehyde-deamino-DPN	0

tube, a liquid N_2 trap and counted, a small amount of radioactivity was found in the gas sample. No quantitative data can be given owing to the limitations of the radioactive counter and other equipment.

Under conditions described in Table III, tritium ions exchange with a non-volatile compound assumed to be DPNH. The rates of exchange are approximately

TABLE III

TRITIUM INCORPORATION FROM MEDIUM

The incubation mixture contained, in a volume of 3.0 ml, 2 μmoles DPNH or 2 μmoles DPN and the reaction mixture saturated with H_2; 0.2 unit of dehydrogenase; 300 μmoles Tris–HCl buffer (pH 7.8) or 300 μmoles Tris-succinate (pH 6.9); HTO to give a final specific activity of $1.3 \cdot 10^4$ counts/min/atom H and, when added, 3 μmoles sodium arsenite. Reaction run at 25° for 20 min and stopped by heating on planchettes. Specific activity calculated from concentration of DPNH as determined from absorbancy at 340 mμ.

Conditions	Specific activity (counts/min/μmole)
H_2 + DPN (pH 7.8)	12 500
DPNH (pH 7.8)	12 600
DPNH (pH 6.9)	11 300
DPNH (pH 7.8) + arsenite	12 400

the same whether DPNH is added or generated from H_2 and DPN. Arsenite has no effect on the exchange, which indicates that the small diaphorase contaminant is not catalyzing the exchange reaction.

Reactions in deuterium oxide

The hydrogen dehydrogenase reaction was carried out in the presence of 90% D_2O. The rate of the forward reaction was found to be the same as in water. The reverse reaction, on the other hand, was affected by the presence of D_2O (Fig. 5). The ratio of initial velocities (V_{H_2O}/V_{D_2O}) for 90% D_2O is 2.35.

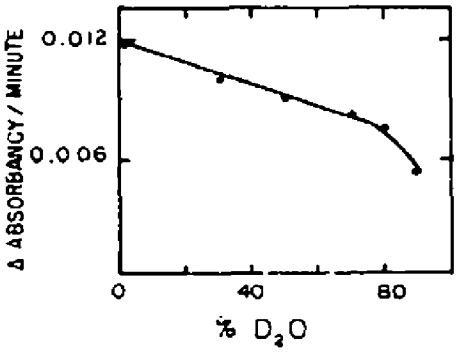

Fig. 5. Effect of deuterium oxide on reverse reaction of hydrogen dehydrogenase. Reaction mixture contained 0.2 μmole DPNH, 100 μmoles Tris succinate (pH 6.9) and 4 units of enzyme in a total volume of 1.0 ml.

Stereochemistry of reaction

The isotope used to determine the stereochemistry of hydrogen dehydrogenase reaction was T_2 gas. After the T_2 had enzymically reduced DPN, DPNT was oxidized with glutamic dehydrogenase, a β-stereospecific enzyme[18], and malic dehydrogenase,

TABLE IV

STEREOSPECIFICITY OF TRITIUM REDUCTION OF DPN

2 ml of solution containing 5 units of hydrogen dehydrogenase, 6 μmoles DPN and 20 μmoles Tris-HCl buffer (pH 7.8) were placed on top of the glass seal of the vial which contained approx. 0.9 μmole tritium gas (50 mC) in 1.0 ml. The rest of the container was flushed with helium and then sealed with a glass stopper. Reaction started by breaking vial seal and incubated for 10 min. Contents were removed, placed in a tube containing 40 μmoles DPNH and then heated to 80° for 2 min. Reaction mixture cooled and centrifuged. Supernatant lyophilised, redissolved in water and used as source of DPNT. The malic dehydrogenase system contained in 1.0 ml malic dehydrogenase, 4 μmoles DPNT, 50 μmoles Tris-HCl (pH 7.8) and oxalacetate added as 3 × 5-μmoles aliquots. Reaction was completed in 20 min as determined at 340 mμ. For analysis of products, 10 μmoles DPN and 10 μmoles malate were added to the reaction mixture, cooled and protein precipitated by adding $HClO_4$ to a final concentration of 0.2 M. Mixture was centrifuged and the supernatant was neutralized with 1 M KOH. After the $KClO_4$ had precipitated, the supernatant was applied to a 7 × 0.9 cm column of Dowex-1 formate[19] and the column was then washed with 50 ml of water. DPN and the acid were eluted with a linear gradient between 50 ml water and 50 ml 3 N formic acid; 2-ml fractions were collected. The glutamic dehydrogenase reaction mixture contained in 1.0 ml: glutamic dehydrogenase, 15 μmoles α-ketoglutarate, 15 μmoles NH_4Cl, 50 μmoles Tris HCl (pH 7.8) and 4 μmoles DPNT. Reaction ran to completion in 20 min. 10 μmoles glutamic acid and 10 μmoles DPN were then added. DPN and glutamic acid were separated as described for malic dehydrogenase.

	Malic dehydrogenase		*Glutamic dehydrogenase*	
	Malate	*DPN*	*Glutamate*	*DPN*
Radioactivity	-	+	-	+

an α-stereospecific enzyme[19]. Radioactivity was found in glutamic acid and in DPN produced in the malic dehydrogenase reaction (Table IV) which means that hydrogen dehydrogenase has β-stereospecificity. This method of using α- and β-stereospecific enzymes to determine the stereospecificity of addition of hydrogen to TPN has been applied to several enzymes[20-22].

DISCUSSION

Cyanide has been the only inhibitor of hydrogenases which has been studied by several workers[1,2,5,9,14,23]. Cyanide has been shown to inhibit the exchange reaction of the hydrogenase of *Proteus vulgaris* but only when the enzyme is oxidized[1,9]. Similarly, the oxidized form of *Azotobacter vinelandii* hydrogenase is inhibited by cyanide[23]. With highly purified preparations of hydrogenase from *D. desulfuricans* there exists a discrepancy in the effects of cyanide[2,14]. These differences could be attributed to the different assay conditions. (a) Exchange reaction at pH 6.7: cyanide (10^{-3} M) inhibits the oxidized form 25% (see ref. 2). (b) Methylene blue as an hydrogen acceptor at pH 8.4: cyanide (10^{-3} M) inhibits 100%, independent of the state of the enzyme, *i.e.* oxidized or reduced[14].

The more detailed studies with cyanide and other inhibitors on hydrogen dehydrogenase of *H. ruhlandii* (Table I) indicate that their effect is likely to be due to the splitting of either a disulfide bond or perhaps a metal sulfur bridge such as is postulated for xanthine oxidase[24]. That iron is part of the mechanism of hydrogen activating enzymes has been indicated by the presence of iron in a highly purified preparation of hydrogenase of *D. desulfuricans*[4] and by a requirement for ferrous

iron for maximum activity of the enzymes from *Clostridium butylicum*[6] and *D. desulfuricans*[8]. Ferrous iron is also implicated in systems reducing DPN[5,25,26]. Work on *Pseudomonas saccharophila* suggests that iron is linked to the protein by a thiol group[25]. A fuller description of the bond being split will require data from quantitative studies.

On reduction of hydrogen dehydrogenase of *H. ruhlandii* with H_2 or DPNH, the bond is split and produces a PCMB sensitive thiol group(s). The lack of inhibition by arsenite suggests that a vicinial dithiol is not produced on reduction of the enzyme. The reduced bond is assumed to react with DPN to give DPNH, which would be in accord with cyanide, sulfide and sulfite acting as competitive inhibitors of DPN (Fig. 1). Kinetic analysis also supports this type of mechanism involving the reduction–oxidation of a sulfur bridge and has been formulated as Eqns. 1 and 2.

This mechanism could explain the inhibitory action of oxygen on hydrogenases of anaerobic bacteria which has previously been interpreted as oxygenation and oxidation of the enzyme[27]. Oxygenation inhibition was found to be reversed on removal of oxygen[27] and this inhibition can now be accounted for as the oxidation of thiol group(s) which are generated on reduction of the enzyme by H_2.

There is an analogous enzyme, the flavoprotein dihydrolipoyl dehydrogenase, which shows the same kinetics[29] as Fig. 3 for hydrogen dehydrogenase and has a disulfidedithiol group as an intermediate[30,31]. This flavoprotein also catalyzes an exchange reaction between DPNH and deuterium oxide[32]. Several of the flavoprotein dehydrogenases show varying exchange rates[22,32,33] and in one case there is no exchange[34]. Flavoproteins are not unique in promoting an exchange between a reduced substrate and water. The hydrogenase of *D. desulfuricans* carries out an exchange between the enzyme hydride and deuterium oxide, concurrently with the reversal of a reaction like Eqn. 1. This results in the appearance of two species of hydrogen *i.e.* DD and HD and the relative rate of formation of DD to HD is 1.1 (see ref. 2). The experiments with tritium isotope and hydrogen dehydrogenase do not distinguish between reaction 1 and the exchange between the enzyme and water. As tritium is incorporated rapidly into DPNH from HTO, it can be concluded from the appearance of labelling only in the β-position of DPNH, that the stereospecificity of the exchange and dehydrogenase reactions are the same.

The removal of the lag phase by preincubating the enzyme with H_2 or DPNH could be explained by the existence of two enzymic forms, one being inactive which is in equilibrium with the active form. On reduction of the active form, the inactive form is slowly converted into the active form. This lag phase contrasts with that described by WITTENBERG AND REPASKE[35] for extracts of *H. eutropha* which reduced DPN with H_2 and had a lag phase that was not affected by DPNH or H_2. The effect of sulfide also distinguishes the DPN reducing systems in *H. eutropha* and *H. ruhlandii* where in the former sulfide stimulates activity[36] and in the latter is a strong inhibitor (Table I).

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