where the terms are defined by eq A4–A6. The total succinylation stoichiometry is the sum of the contributions from the two parallel paths, $y_1 + y_2$.

The total number of active $E_1$ subunits per complex, $N_1 + N_2$, is assumed to be 12 for the unmodified complex, or to be reduced proportionally when the $E_1$ activity is reduced with MalNEt. The fitting procedure used distributes these subunits between the two classes described above to match the observed limiting succinylation stoichiometry, while simultaneously fitting $k_1$ and $k_2$. For example, this mechanism would account for the succinylation of 15 lipoic acids by 12 $E_1$ subunits by requiring that 9 $E_1$ subunits succinylate 1 lipoic acid per $E_1$ and 3 $E_1$ subunits succinylate 2 lipoic acids per $E_1$. After the values of $N_1$ and $N_2$ were fixed, the time courses were fit directly to eq A2 and A7.

**Registry No.** MalNEt, 128-53-0; $E_1$, 37205-42-8; α-ketoglutarate dehydrogenase, 9031-02-1; α-ketoglutaric acid, 328-50-7.

References


Akiyama, S. K. (1980) Ph.D. Thesis, Cornell University, Ithaca, NY.

Akiyama, S. K., & Hammes, G. G. (1980) *Biochemistry 19*, 4208–4213.

Ambrose-Griffin, M. C., Danson, M. J., Griffin, W. G., Hale, G., & Perham, R. N. (1980) *Biochem. J. 187*, 393–401.

Angelides, K. J., & Hammes, G. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4877–4880.

Angelides, K. J., & Hammes, G. G. (1979) *Biochemistry 18*, 5531–5537.

Collins, J. H., & Reed, L. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4223–4227.

Danson, M. J., Fersht, A. R., & Perham, R. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5386–5390.

DeRosier, D. J., Oliver, R. M., & Reed, L. J. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1135–1137.

Hackert, M. L., Oliver, R. M., & Reed, L. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2226–2230.

Koike, M., Reed, L. J., & Carrol, W. R. (1963) *J. Biol. Chem. 238*, 30–39.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Pettit, F. H., Hamilton, L., Munk, P., Namihira, G., Eley, M. H., Willms, C. R., & Reed, L. J. (1973) *J. Biol. Chem. 248*, 5282–5290.

Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40–46.

Speckhard, D. C., Ikeda, B. H., Wong, S. S., & Frey, P. A. (1977) *Biochem. Biophys. Res. Commun. 77*, 708–713.

Von Korff, R. W. (1969) *Methods Enzymol. 13*, 519–523.

Wagenknecht, T., Francis, N., & DeRosier, D. J. (1983) *J. Mol. Biol. 165*, 523–541.

Waskiewicz, D. E., & Hammes, G. G. (1982) *Biochemistry 21*, 6489–6496.

White, R. H., Bleile, D. M., & Reed, L. J. (1980) *Biochem. Biophys. Res. Commun. 94*, 78–84.


# Isotope, Pulse–Chase, Stopped-Flow, and Rapid Quench Studies on the Kinetic Mechanism of Bovine Dihydropteridine Reductase†

Susmita Poddar‡ and Jack Henkin*

ABSTRACT: The kinetics of the reduction of quinonoid 2-amino-4-hydroxy-6,7-dimethyldihydropteridine ($DMPH_2$) catalyzed by bovine liver dihydropteridine reductase were examined with NADH, (*S*)-NADD, (*S*)-NADT, and [$^3$H]-NADH as substrates. No significant deuterium isotope effect was observed on either $K_m$ or $V_m$, indicating that hydrogen transfer is not a major rate-limiting step of the reaction. Tritium from (*S*)-NADT is transferred to an exchangeable position of the pteridine product without significant isotopic discrimination. The ratio of tritium released into solvent to $NAD^+$ produced is approximately 1.0 in the steady state as well as in the first enzyme turnover as determined by pulse–chase experiments. Pulse–chase methods also showed that the binary complex E·NADH is fully functional and can be completely converted to products prior to NADH dissociation in the presence of saturating $DMPH_2$. The concentration of $DMPH_2$ giving half-maximal trapping of E·NADH is identical with its $K_m$ as determined by steady-state kinetics. Stopped-flow kinetic measurements gave no evidence for a burst of NADH utilization. This was further demonstrated by rapid quench experiments which demonstrated a pre-steady-state rate nearly identical with that of the steady state. The above results are consistent with nonequilibrium ordered binding of substrates and with a rate-limiting isomerization in the ternary complex which precedes hydrogen transfer.

Dihydropteridine reductase (DHPR)[1] (EC 1.6.99.7) reduces the quinonoid dihydropteridine formed during hydroxylation of aromatic compounds (Kaufman & Fisher, 1974). Kinetic analysis of the bovine liver enzyme (Chauvin et al., 1979; Asknes & Ljones, 1980) using quinonoid 6,7-dimethyldihydropterin ($DMPH_2$) as a substrate, the observation that one NADH per monomer is tightly bound (Hasegawa, 1977; Webber & Whiteley, 1978; Chauvin et al., 1979), and results with affinity chromatography (Korri et al., 1977; Chauvin et

† From the Department of Biochemistry and Molecular Biology, The University of Texas Medical School Health Science Center, Houston, Texas 77225. *Received November 17, 1983.* This work was supported in part by Grant AU 826 from the Robert A. Welch Foundation.

‡ Recipient of a Rosalie B. Hite Fellowship.

[1] Abbreviations: DHPR, dihydropteridine reductase; $DMPH_2$, quinonoid 2-amino-4-hydroxy-6,7-dimethyldihydropteridine; $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; $NAD^+$, nicotinamide adenine dinucleotide; [$^3$H]$NAD^+$, $NAD^+$ with general tritium label in the adenosine ring; NADH, reduced nicotinamide adenine dinucleotide; NADD, 4-deuterio-NADH; NADT, 4-tritio-NADH; [$^3$H]NADH, NADH with general tritium label in adenosine ring; NAD* or NADH*, $NAD^+$ or NADH radioactively labeled; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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al., 1979) are consistent with a compulsory order mechanism, with NADH being the leading substrate. The product, $NAD^+$, is competitive with NADH, while the dihydropterin substrate ($DMPH_2$) and $NAD^+$ are noncompetitive (Asknes & Ljones, 1980), suggesting that $NAD^+$ dissociates last. Armarego (1979) has shown that the *pro-S* hydrogen of NADH is specificially transferred to an exchangeable position on the reduced pteridine and rapidly equilibrates with water.

In the following study we describe kinetic, pulse–chase, stopped-flow, and rapid quench experiments with DHPR from beef liver to examine the properties of the E·NADH complex and to ascertain whether there is any primary kinetic isotope effect or isotope discrimination in this enzyme-catalyzed hydrogen transfer. The results are consistent with a compulsory ordered kinetic mechanism in which the rate-determining step precedes the chemical step.

## Materials and Methods

2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) was obtained from Aldrich. $NAD^+$ and NADH were purchased from P-L Biochemicals. $NAD^+$ ([2,8-$^3$H]-adenine) and D-[1-$^3$H]glucose were from New England Nuclear. [$^2H_6$]Ethyl alcohol was from MSD Isotopes, and D-[1-$^2$H]glucose from Stohler Isotopes. Glucose-6-phosphate dehydrogenase and yeast alcohol dehydrogenase were obtained from Sigma. Porcine heart lactate dehydrogenase (500–550 units/mg) was also from Sigma. DHPR was isolated and purified by the method of Asknes et al. (1979) from fresh bovine liver. Enzyme activity was measured spectrophotometrically at 27 °C by the oxidation of NADH observed at 340 nm with a Beckman spectrophotometer equipped with a recorder. $K_3Fe(CN)_6$ was used to oxidize $DMPH_4$ (Archer & Scrimgeour, 1970; Nielsen et al., 1969). The reaction mixture (1.0 mL) contained 37.5 mM Tris-HCl, pH 7.8, 300 $\mu$M $K_3Fe(CN)_6$, 10 $\mu$M $DMPH_4$, 100 $\mu$M NADH, and sufficient enzyme to cause a decrease of about 0.05–0.1 absorbance unit/min at 340 nm. The background reaction without enzyme was less than 5% of the enzyme activity, and there was no net decrease in absorbance with enzyme when $DMPH_4$ was omitted. The reaction was followed for a period of about 2 min, and the change in absorbance was linear with time.

(*S*)-NADT and (*S*)-NADD were prepared according to Viola et al. (1979) and [$^3$H]NADH was prepared according to the method of Rafter & Colowick (1957). These were purified by HPLC using a reverse-phase phenyl-$C_{18}$ $\mu$Bondapak column eluting with a gradient from 0 to 40% acetonitrile in 10 mM ammonium acetate buffer. The specific activity of the (*S*)-NADT was 2.38 and that of the [$^3$H]-NADH was 0.96 $\mu$Ci/$\mu$mol.

For pulse–chase experiments, 7.0 nmol of DHPR was treated with 10 nmol of labeled NADH, either (*S*)-NADT or [$^3$H]NADH in 0.5 mL of Tris-HCl, pH 7.8. The excess ligand was removed by dialysis for 12 h. Remaining radioactivity was used to estimate the concentration of DHPR in a 1:1 complex with labeled NADH. The chase solution contained 37.5 mM Tris-HCl, pH 7.8, 300 $\mu$M $K_3Fe(CN)_6$, 466 $\mu$M NADH (500–fold excess over E·NADH), and varying amounts of $DMPH_2$ (derived from added $DMPH_4$) in a total volume of 1.5 mL. This vigorously stirred solution was pulsed with 100 $\mu$L of the E·NADH* solution. Quenching was carried out within 1–2 s with 28% $NH_4OH$ to a final volume of 2.0 mL. Depending on whether (*S*)-NADT or [$^3$H]NADH was used, the subsequent analysis varied as follows:

In the case of (*S*)-NADT, the solution was shell frozen after standing at 25 °C for 10 min. It was then distilled in vacuo in a bulb-to-bulb apparatus with water collected in a cooled receiving flask. Tritium content of an aliquot of the distilled water was determined by scintillation counting as well as in an aliquot of the residue redissolved in 1.0 mL of water.

In the case of [$^3$H]NADH, 0.70 $\mu$mol of unlabeled carrier $NAD^+$ was added (100-fold molar excess over labeled NADH) to the reaction mixture after quenching. The mixture was lyophilized and the residue taken up in 0.5 mL of water. The pyridine nucleotides in the reaction mixture were then separated by HPLC. An aliquot of the $NAD^+$ peak obtained was counted, and the concentration of $NAD^+$ was determined by observing the increase in absorbance at 340 nm when an aliquot was reacted with lactic acid and lactic dehydrogenase (Klingenberg, 1970) by using an extinction coefficient value for NADH of $\epsilon'_{340} = 6.2$ cm$^2$/$\mu$mol. As there is a great excess of added carrier $NAD^+$ over any produced enzymatically, the total cpm in $NAD^+$ was obtained by multiplying the cpm in the HPLC peak by the ratio of carrier $NAD^+$ added to $NAD^+$ found in the counted aliquot.

Rapid quench studies were carried out by using an apparatus identical with that described by Ballou & Palmer (1974) with the flywheel operating at 2400 rpm. The 1:1 complex of E·[$^3$H]NADH was formed as described above at a concentration of 3.0 $\mu$M and was placed in the first syringe. The second syringe contained 50 $\mu$M $DMPH_4$ and freshly prepared 300 $\mu$M $K_3Fe(CN)_6$ in the same buffer. For each reaction 0.5 mL of reactants from each syringe was simultaneously injected through the mixing chamber and into a stirring quench solution consisting of 2.0 mL of 10 M ammonium hydroxide. An accurately known molar excess of unlabeled carrier $NAD^+$ (0.30 $\mu$mol) was then added to each quenched reaction, and the mixtures were stored frozen at −90 °C. Aliquots of each were applied to a 1 × 10 cm column of Dowex 1-formate (Pallini & Ricci, 1965). $NAD^+$ eluted well ahead of NADH when 50 mM ammonium formate buffer, pH 8.0, was used as solvent. [$^3$H]$NAD^+$ was assayed with LDH and counted as described above to calculate [$^3$H]$NAD^+$ production. No [$^3$H]$NAD^+$ formation was detected when the syringes were fired in rapid succession rather than simultaneously indicating complete quenching. The quenching time of the instrument was estimated under similar conditions using porcine lactate dehydrogenase (LDH). This enzyme has a very high turnover rate and is known to have equal rates in the pre steady and steady states in the direction of lactate formation (Stinson & Gutfreund, 1971). Thus, in a calibration experiment syringe 1 contained 75–150 units of LDH and 28 $\mu$M NADH in Tris buffer, pH 7.8; syringe 2 contained 1.0 mM sodium pyruvate. Rapid mixing (0.5 mL each) and quench were carried out into 2.0 mL of 10 M $NH_4OH$ in 8 M urea. This was required to prevent additional reaction in the quench mixture. The amount of NADH consumed was assayed spectrophotometrically at 340 nm and compared with controls where mixing was carried out slowly by hand or in advance of quenching or where the syringes were fired successively in either order. No reaction was detected in the latter case. When the steady-state rate for LDH was measured and the same rate was assumed during the mixing time, the quenching time was estimated to be 3.5 ± 1 ms.

Stopped-flow fluorometric studies were performed with an instrument similar to that described by Nall & Landers (1981). The mixing ratio was 1:1, and the excitation and emission wevelengths were 340 and 470 nm, respectively. All kinetic studies were carried out in 50 mM Tris-HCl (pH 7.8). Syringe 1 contained 25 $\mu$M or 25 nM DHPR and 10 $\mu$M NADH while syringe 2 contained 50 $\mu$M $DMPH_4$ and freshly prepared 300 $\mu$M $K_3Fe(CN)_6$ in the same buffer. Fluores-

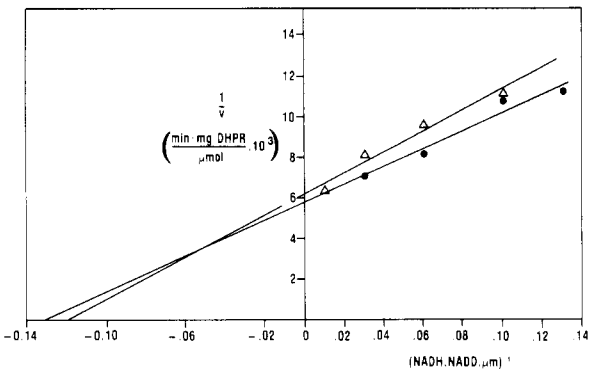



FIGURE 1: Double-reciprocal plot (Lineweaver & Burk, 1934) with varying concentrations of (△) NADH and (●) NADD. Assay was carried out with 10 $\mu$M $DMPH_2$ as described under Materials and Methods.

cence was converted into a proportional millivolt output. A slow continuous linear drift of decreasing fluorescence, and of unknown origin, was observed with high enzyme concentrations (Figure 4B). The drift was subtracted to correct the overall millivolt change at $t = 0.45$ s.

## Results and Discussion

Kinetic constants obtained from initial velocity studies using $K_3Fe(CN)_6$ to oxidize $DMPH_4$ to $DMPH_2$ were as follows: $K_{DMPH_2} = 7.1$ $\mu$M, $K_{NADH} = 8.7$ $\mu$M, and $V_{max} = 161$ $\mu$mol $min^{-1}$ $mg^{-1}$; the turnover number was 3600 $min^{-1}$.

Use of either the DHPR·NADH binary complex as isolated after affinity chromatography or exhaustively dialyzed NADH-free DHPR gave the same values. The kinetic constants compare favorably with those determined by Asknes & Ljones (1980) (7.3 $\mu$M, 9.1 $\mu$M, and 167 $\mu$mol $min^{-1}$ $mg^{-1}$, respectively) even though $H_2O_2$/peroxidase, rather than ferricyanide, was used for a dihydropteridine recycling system by those workers. The values were similar to those found for other mammalian DHPRs (Craine et al., 1975; Cheema et al., 1973).

*Isotope Effects.* Deuterated NADH (NADD) was compared as a substrate with NADH (Figure 1) by using $DMPH_2$ at a concentration of 10 $\mu$M (1.5$K_m$ value). Varying the amount of NADH and (4*S*)-[4-$^2$H]NADH (NADD) as shown in double-reciprocal form indicates no effect on $V_{max}$ within experimental error. There also was no significant difference in $K_m$ values for the pyridine nucleotides ($V_H/V_D = 0.94 \pm 0.1$; $K_H/K_D = 1.1 \pm 0.1$). C–H bond breaking cannot, therefore, be the rate-determining step under steady-state conditions. A small equilibrium isotope effect on the distribution of species preceding the rate-determining step cannot be ruled out.

*Tritium Selection.* Isotopic discrimination was examined by using the same conditions as above, but with (4*S*)-[4-$^3$H]NADH (NADT). It has been shown (Armarego, 1979) that this tritium is transferred to an exchangeable position of the tetrahydropteridine ($DMPH_4$) product and, therefore, appears totally as tritiated water. Under steady-state conditions we did not observe $^3$H selection; $1.0 \pm 0.05$ atoms of tritium was released per molecule of $NAD^+$ formed (Figure 2). Thus, no enrichment of the substrate or rapid washout of tritium takes place. Although no primary isotope discrimination was detected, a very small equilibrium isotopic discrimination cannot be ruled out.

Two likely explanations are possible: (a) Bound NADH in the ternary complex is committed to go on to products; i.e. $E{\cdot}^{NADH}_{DMPH_2}$ goes forward to products much more often than dissociating to yield free NADH. The rate-determining step may

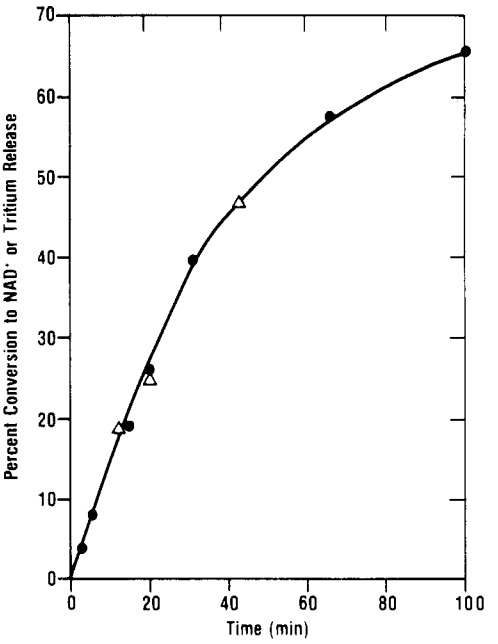



FIGURE 2: Tritium isotope effect. (●) Reaction followed by measuring disappearance of NADH; (△) distilled counts obtained from $NH_4OH$-quenched reaction at appropriate time intervals.

be either before or after hydrogen transfer. (b) NADH can dissociate readily from the ternary complex, but all steps up to or including H transfer are at or near equilibrium, with a later step (e.g., product dissociation) being rate determining. Since there is no rapid washout of radioactivity into water, $NAD^+$ formation and $^3$H release are in a 1:1 ratio. Either $^3$H is only released from free $DMPH_4$ (explanation a) or $^3$H is shielded against exchange with water in the enzyme–product complex (explanation b).

*Pulse–Chase Experiments.* In order to determine what fraction of binary E·NADH complex can be transformed into products during the very first enzyme turnover, we employed an analysis described by Rose et al. (1974) in which a pulse solution containing E·NADT was added to a larger volume of rapidly stirring chase solution which contained a 500-fold excess of unlabeled NADH and varying amounts of $DMPH_2$, as described under Materials and Methods. The reaction was quenched in 1–2 s (less than 15 turnovers under the experimental conditions used) by addition of concentrated $NH_4OH$.

Controls were also carried out in which enzyme alone was added to labeled NADH preequilibrated with the carrier NADH or where the reaction was allowed 5 min to go to completion (>98%) before quenching. The fraction of the total tritium added which was released into water was dependent on the concentration of $DMPH_2$ present in the chase (Figure 3), and it extrapolated to complete (>95%) trapping of the E·NADT complex in the first turnover at infinite $DMPH_2$ concentration.

It is conceivable that in these experiments $^3$H could have been released from enzyme-bound $DMPH_4$ at a greater rate than the formation of free products. Therefore, a pulse–chase study, as above, was also carried out by using E·NADH where the cofactor was tritiated in the adenine moiety. An isotopic dilution method was applied in order to quantitate E·NADH* trapping. An accurately known amount of $NAD^+$ (700 nmol, 100-fold excess unlabeled $NAD^+$ over the total NADH delivered in the pulse) was added as carrier after $NH_4OH$ quenching (see Materials and Methods). The fraction of E·NADH* trapped in the first turnover measured as NAD* was exactly the same (Figure 3) as that found above by

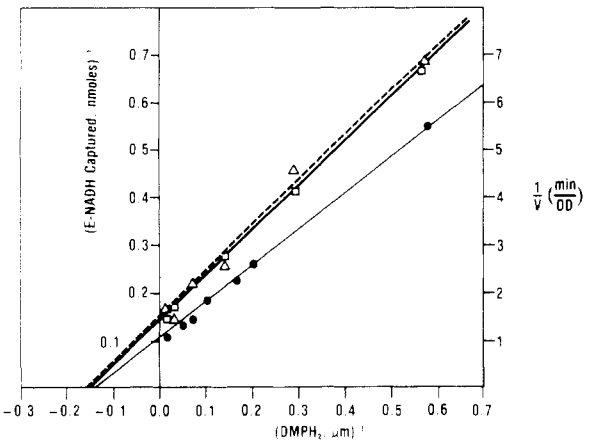



FIGURE 3: Pulse–chase experiments. E·NADH is trapped as a function of $DMPH_2$ concentration. (△) [$^3$H]NAD derived from E·[$^3$H]NADH was determined as described under Matierals and Methods. (□) $^3$H release into water from E·NADT (see Materials and Methods). (●) Steady-state rate as measured by $\Delta OD_{360}$ ($OD_{340}$ was too high for accurate measurement). The $K_m$ under pulse–chase conditions is shown to be equal to $K_{1/2}$.

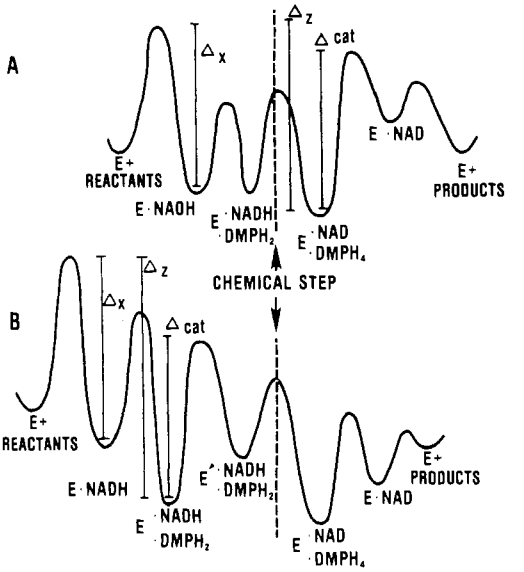
measuring $T_2O$. Thus, the 1:1 correspondence of $^3$H release to free $NAD^+$ formation also holds true for the first enzyme turnover. This indicates that the partitioning of E·NADH is not controlled by a hydrogen-transfer step in the first turnover, as such a case would predict a smaller forward partitioning for NADT than for [$^3$H]NADH. The $K_m$ of $DMPH_2$ was measured by steady-state kinetics carried out under conditions identical with those of the pulse–chase (Figure 3), i.e., with high NADH concentrations. The value of $K_m$ for $DMPH_2$ was identical with that obtained under normal steady-state conditions (although $V_m$ is reduced by very high NADH concentration) and also was found to be identical with the concentration of $DMPH_2$ required for half-maximal trapping ($K_{1/2}$) of E·NADH in a single turnover (Rose et al., 1974) (Figure 3).

The substantial trapping obtained in the pulse–chase clearly shows that E·NADH is a fully functional binary complex. The observation that all of this complex can be trapped by saturating concentrations of $DMPH_2$ is consistent with the compulsory order of binding forwarded from kinetic studies (Asknes & Ljones, 1980) although it is not possible, in general, to rule out a random order of binding where the ternary complex is extremely tightly bound and always goes on to products. Since trapping of the ternary complex is so efficient, the binding of substrates cannot be at equilibrium, at least not for the first turnover. It follows that to the extent that bound NADH was not captured as product in the first turnover; i.e., when $DMPH_2$ was below saturating concentration, NADH must have dissociated exclusively (>95%) from the binary complex. This allows an analysis (Rose et al., 1974) of the dissociation rate constant $k_x$ for E·NADH, viz.

$$k_x = \frac{K_{1/2}k_{cat}}{K_m}$$

where $k_{cat}$ is $V_m/E$ in the steady state. Since we find that $K_{1/2} = K_m$ for DHPR, it follows that $k_x = k_{cat}$. The assumption that $k_{cat}$ as used above can always be taken as $V_m/E$ in the steady state is not always applicable. One can postulate, for example, a compulsory ordered release mechanism where dissociation of the second product is rate determining in the steady state but where the dissociation of the first product is rapid and makes the first turnover complete and irreversible at a rate greater than the apparent $k_{cat}$. Thus, $k_x$, as has been pointed out by Rose et al. (1974), is a minimum value for the

Scheme I




rate constant of E·S dissociation in the general case. In the present case, however, $NAD^+$ is a poor inhibitor ($K_i$ = 1.1 mM) and probably dissociates last and rapidly (Asknes & Ljones, 1980). Rather, if product release is rate controlling, it would be more likely that all steps between $E{:}^{NADH}_{DMPH_2}$ and $E{:}^{NAD^+}_{DMPH_4}$ are near equilibrium before relatively slow $DMPH_4$ dissociation. Such a case is illustrated in the free energy diagram of Scheme IA. If $E{:}^{NAD^+}_{DMPH_4} \rightarrow E{\cdot}NAD^+ + DMPH_4$ is rate determining for free product ($NAD^+$) formation, in both the transient state and the steady state, the above equation is valid and $k_x = k_{cat} = V_m/E$ in this case. The lack of isotope discrimination or kinetic isotope effect is consistent with such a rate-determining step, although it is impossible to rule out, say, rate-determining enzyme isomerization subsequent to product release. Another possible rate-determining step which is consistent with the observations described thus far and with the above equation is a slow isomerization of the substrate ternary complex prior to hydrogen transfer (Scheme IB). In this case the rate-determining step would be identical for both the steady and transient state.

*Stopped-Flow Kinetics.* In order to distinguish the above possibilities (Scheme I), stopped-flow kinetic experiments were carried out to ascertain whether any kinetic burst of NADH consumption was detectable. Such a burst would be expected for a rate-determing step subsequent to hydrogen transfer as in Scheme IA but would not be predicted for the case (Scheme IB) where rate-limiting enzyme isomerization precedes hydrogen transfer. NADH and DHPR in a 4:1 ratio, under conditions where nearly all enzyme molecules contained bound NADH, were rapidly mixed with $DMPH_2$ and a $DMPH_4 \rightarrow DMPH_2$ recycling system. Decrease in NADH fluorescence was followed (Figure 4A) from 10 ms after mixing to 1 s. As a control, the same amount of NADH was reacted under identical conditions but with 100-fold less enzyme, with decrease in fluorescence being monitored to completion up to 10 s (Figure 4B). The control establishes the millivolt change expected for utilizing all of the NADH after mixing. If a burst of one turnover occurred within the effective instrument dead time (5 ms) for the experimental mixing (Figure 4A), its apparent millivolt change would be reduced by approximately one-fourth compared with that of the control. Within experimental error the control and experimental mixing show identical (±10%) changes. There was no evidence of any one equivalent burst of NADH utilization or of any biphasic kinetics.

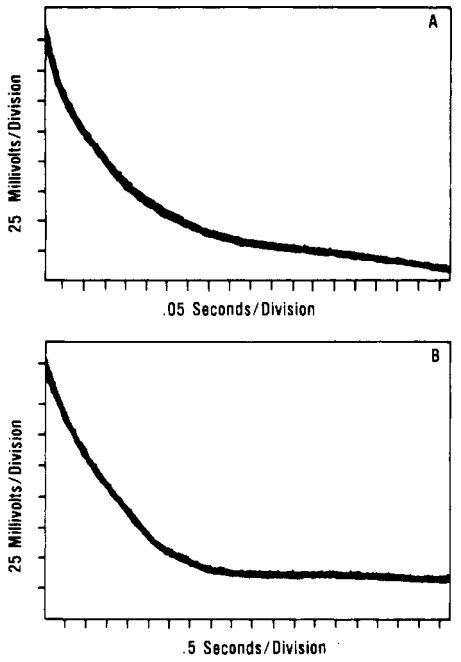



FIGURE 4: (A) Stopped-flow kinetics on rate and extent of reaction of NADH with high concentration of DHPR. Conditions: syringe 1, 2.5 $\mu$M DHPR + 10.0 $\mu$M NADH; syringe 2, 50 $\mu$M $DMPH_4$ + 300 $\mu$M $K_3Fe(CN)_6$, both in 50 mM Tris-HCl buffer (pH 7.8). The reaction is >95% complete in 0.45 s. The total change in fluorescence corresponds to 170 ± 15 mV. If there had been a burst within the experimental dead time, the total observed change in fluorescence would have corresponded to 128 ± 12 mV. The actual change is not less than 160 mV within experimental error. Although the NADH concentration is at $K_m$ value, all the NADH present is bound to the enzyme [i.e., $K_d \ll K_m$ as shown by Asknes et al. (1979)]. (B) Rate and extent of NADH utilization with low concentration of DHPR. Conditions: syringe 1, 0.025 $\mu$M DHPR + 10.0 $\mu$M NADH; syringe 2, 50 $\mu$M $DMPH_4$ + 300 $\mu$M $K_3Fe(CN)_6$, both in 50 mM Tris-HCl buffer (pH 7.8). The total change in fluorescence corresponds to 165 ± 10 mV.

Confidence in the above stopped-flow results is limited by the low accuracy of measuring a relatively small burst of fluorescence loss within the instrumental dead time and in the presence of a 4-fold excess of NADH, with substantial background noise. To further test the possibility of a burst, E·[$^3$H]NADH was reacted with saturating concentrations of $DMPH_2$ in a rapid quench apparatus as described under Materials and Methods. Calibration using lactic dehydrogenase showed that components were free to react for 3.5 ± 1 ms before quenching under the experimental conditions. If E·[$^3$H]NADH reacted at its steady state $k_{cat}$ (3600 $min^{-1}$) only about 17% should be converted into [$^3$H]$NAD^+$ during the experiment. A much greater amount of [$^3$H]$NAD^+$ (up to 1 equiv) would be formed as already shown in the pulse–chase experiments in the event of a faster burst rate for the first enzyme turnover. The results were that the E·[$^3$H]NADH complex gave 4590 dpm as [$^3$H]$NAD^+$ when reacted to completion (10s) but only 780 dpm as [$^3$H]NAD when reacted by rapid quench (3.5 ms). This is precisely 17% of the complete reaction, indicating again that there is no rapid first turnover. Rather, the first turnover rate is identical with that measured in the steady state.

The lack of a deuterium isotope effect precludes hydrogen transfer as the rate-determining step. With no evidence for a burst, it must also be concluded that the rate-determining step precedes the chemical step. This is most consistent with the free energy diagram of Scheme IB. Another argument favoring a kinetic mechanism like B rather than A is that no tritium washout was observed in either the first turnover or in the steady state. This is consistent with a high degree of commitment to catalysis (Northrop, 1975) in the substrate ternary complex and, unlike mechanism A where the substrate and product ternary complexes can interconvert often before $DMPH_4$ dissociation, does not require postulation of a sequestered proton to explain the lack of tritium washout. It should be pointed out, however, that mechanism A can be varied so as not to require proton shielding by substantially lowering the energy of $E{:}^{NAD^+}_{DMPH_4}$ and equally lowering the subsequent energy barrier in going to E·$NAD^+$. This makes the chemical step essentially irreversible although not rate determining. Indeed, all of our attempts to observe the reverse reaction were unsuccessful. To be consistent with our finding that $K_{1/2} = K_m$, the height of the energy barrier for hydrogen transfer would have to be equal to $\Delta_x$ for E·NADH dissociation in such a case. This variant of mechanism A also predicts a burst of NADH utilization and is thus inconsistent with our results.

According to part A or B of Scheme I, at saturating $DMPH_2$ nearly all bound NADH will go forward to products since the free energy barrier $\Delta_{cat}$ is smaller than $\Delta_z$. This is consistent with the observed complete trapping of E·NADH in the presence of saturating levels of $DMPH_2$ in the first enzyme turnover. From the lack of an isotope effect and of any burst in the transient phase, it is concluded that the rate which controls $k_{cat}$ is an isomerization of the ternary complex preceding hydrogen transfer as indicated in Scheme IB. A similar rate-determining step was deduced by Stinson & Gutfreund (1971) from their transient state kinetic and isotopic studies on porcine heart lactic dehydrogenase. The rate of the reduction of pyruvate by NADH catalyzed by that enzyme is also controlled by a slow isomerization prior to the chemical step.

In both diagrams (A and B) $\Delta_x$ and $\Delta_{cat}$ have been set equal in order to accommodate the conclusion that $k_x = k_{cat}$ as determined by the observation that $K_{1/2} = K_m$. In setting these energy barriers equal, it is assumed that all enzyme-bound species up to the rate-determining step are distributed at their steady-state levels. Thus, when the concentration of $DMPH_2$ is equal to its $K_m$ value, the complexes E·NADH and $E{:}^{NADH}_{DMPH_2}$ should be equally populated to give a velocity equal to $1/2V_M$. It is interesting to speculate as to why these two rates ($k_x$ and $k_{cat}$) are equal other than by pure coincidence. Perhaps a slow conformational change that exposes bound NADH for escape to solvent is identical with that required in the ternary complex in order to allow proper alignment of the pteridine substrate in relation to the pyridine nucleotide for chemical reaction. This might coincide with or be controlled by a relatively slow enzyme ionization or proton transfer (Cook & Cleland, 1981).

The energy barrier between the binary and ternary complexes of enzyme and substrates in Scheme IB can be overcome in the forward direction by sufficiently high concentrations of $DMPH_2$. Its height is set to reflect the near irreversibility of $DMPH_2$ binding relative to the subsequent forward reaction, consistent with the high degree of E·NADH capture as observed in pulse–chase experiments. This, along with other observations, agrees with a nonequilibrium binding mechanism. For example, the kinetic double-reciprocal plots of Asknes & Ljones (1980) indicate ordered substrate binding but do not intersect at the vertical axis as would be expected in the case of a rapid equilibrium ordered mechanism (Fromm, 1975). Furthermore, the dissociation constant, $K_d$, for the binary complex E·NADH has been directly measured at pH 7.8 to be approximately $4.2 \times 10^{-8}$ M (Lind, 1973; Asknes et al., 1979) while $K_m$ for NADH at saturating $DMPH_2$ concen-

trations is $8.7 \times 10^{-6}$ M. The 200-fold discrepancy between $K_d$ and $K_m$ suggests a very strong forward commitment to reaction. If $K_d = 4.2 \times 10^{-8}$ M and also equals $k_x/k_{on}$, then $k_{on}$ for NADH is calculated by using $k_x = k_{cat} = 60$ s$^{-1}$ to be near the diffusion-controlled limit at $1.4 \times 10^9$ M$^{-1}$ s$^{-1}$. For the scheme

$$E + NADH \underset{k_x}{\overset{k_{on}}{\rightleftharpoons}} E{\cdot}NADH \underset{k_{-1}}{\overset{k_1(DMPH_2)}{\rightleftharpoons}} E{:}^{NADH}_{DMPH_2} \xrightarrow{k_{cat}} \text{products}$$

$K_{DMPH_2}$ equals $(k_{-1} + k_{cat})/k_1$. Assuming from the above arguments that $k_{-1} \ll k_{cat}$, this reduces to $K_{DMPH_2} = k_{cat}/k_1$. Since $K_{DMPH_2}$ and $k_{cat}$ are respectively known to be $7 \times 10^{-6}$ M and 60 s$^{-1}$, $k_1$ can be calculated as $8.6 \times 10^6$ M$^{-1}$ s$^{-1}$.

The kinetic description of DHPR which emerges from the results of this study is one in which the enzyme combines rapidly with NADH to form a tightly bound fully functional binary complex. This combines more slowly with $DMPH_2$ to form a ternary complex. The most common fate of the latter is to undergo an isomerization, the rate of which controls that of the forward reaction in both the steady and pre steady states under conditions where $DMPH_2$ is at saturating concentrations. The rate of this isomerization is identical with that for dissociation of the binary complex E·NADH and may have some chemical or physical step in common with it. The hydrogen transfer and all subsequent steps are relatively fast and essentially irreversible. Thus, the enzyme is poised to efficiently catalyze product formation every time a molecule of $DMPH_2$ is bound. It would be of considerable interest to ascertain whether similar kinetic behavior is observed when quinonoid dihydrobiopterin, the physiological substrate, is used.

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**Registry No.** $DMPH_2$, 5977-33-3; NADH, 58-68-4; (*S*)-NADD, 10021-11-1; (*S*)-NADT, 6797-57-5; [$^3$H]NADH, 90269-17-3; DHPR, 70851-99-9.

## References

Archer, M. C., & Scrimgeour, K. G. (1970) *Can. J. Biochem. 48*, 278.

Armarego, W. L. (1979) *Biochem. Biophys. Res. Commun. 89*, 246.

Asknes, A., & Ljones, T. (1980) *Arch. Biochem. Biophys, 202*, 342.

Asknes, A., Skotland, T., Flatmark, T., & Ljones, T. (1979) *Neurochem. Res. 4*, 385.

Ballou, D. P., & Palmer, G. A (1974) *Anal. Chem. 46*, 1248.

Chauvin, M. M., Korri, K. K., Tirpak, A., Simpson, R. C., & Scrimgeour, K. G. (1979) *Can. J. Biochem. 57*, 178.

Cheema, S., Soldin, S. J., Knapp, A., Hoffman, T., & Scrimgeour, K. G (1973) *Can. J. Biochem. 51*, 1229.

Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 1805.

Craine, J. E., Hall, E. S., & Kaufman, S. (1972) *J. Biol. Chem. 247*, 6082.

Fromm, H. (1975) *Initial Rate Enzyme Kinetics*, p 37, Springer-Verlag, New York.

Hasegawa, H. (1977) *J. Biochem.* (Tokyl) *81*, 169.

Kaufman, S., & Fisher, D. B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 285–369, Academic Press, New York.

Klingenberg, M. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., Ed.) 2nd ed., p 1975, Verlag Chemie, Weinheim.

Korri, K. K., Chippel, D., Chauvin, M. M., Tirpak, A., & Scrimgeour, K. G. (1977) *Can. J. Biochem. 55*, 1145.

Lind, K. E. (1973) *Eur. J. Biochem. 33*, 67.

Lineweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658.

Nall, B., & Landers, T. (1981) *Biochemistry 20*, 5403.

Nielsen, K. H., Simonsen, V., & Lind, K. E. (1969) *Eur. J. Biochem. 9*, 497.

Northrop, D. B. (1975) *Biochemistry 14*, 2644.

Pallini, V., & Ricci, C. (1965) *Arch. Biochem. Biophys. 112*, 282.

Rafter, G. W., & Colowick, S. P. (1957) *Methods Enzymol. 3*, 887.

Rose, I. A., O'Connell, E. L., Litwin, S., & Bar Tana, J. (1974) *J. Biol. Chem. 249*, 5163.

Stinson, R. A., & Gutfreund, H. (1971) *Biochem. J. 121*, 235.

Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334.

Webber, S., & Whiteley, J. M. (1978) *J. Biol. Chem. 253*, 6724.