

Studies on the Mitochondrial Energy-Linked Pyridine Nucleotide Transhydrogenase[†]

Ronald R. Fisher[‡] and Nathan O. Kaplan*

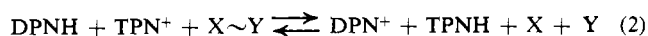
ABSTRACT: The energy-linked pyridine nucleotide transhydrogenase of beef heart submitochondrial particles was studied by the stopped-flow technique. The hydrogen-transfer reaction was assayed in the absence of regenerating systems for either DPNH or TPN⁺ by using the thionicotinamide analog of TPN⁺ (TN-TPN⁺). On addition of respiratory substrate or ATP, a lag of about 30 sec was observed before transhydrogenation proceeded at its maximal rate. The lag was eliminated by preincubating the membranes with energy donor prior to the addition of the substrates. No lag was found in the non-energy-linked DT-transhydrogenase reaction. The maximal rate of the energy-linked transhydrogenase with D₂O was about 50% of that with H₂O with a pD optimum of about 7.8 and a pH optimum of about 7.4. The non-energy-linked transhydrogenase rate was not affected by substituting D₂O for H₂O. The initial transhydrogenase lag in D₂O was nearly

doubled relative to that measured in H₂O. In D₂O, membrane energy-dependent structural changes, indicated by fluorescence enhancement of 8-anilino-naphthalene-1-sulfonic acid, were nearly complete before the onset of transhydrogenation. Dissociation of a proton from a moiety on either the transhydrogenase enzyme or a component of the energy conservation system (having a pK_a of about 7.0) may be required for activation of energy-dependent transhydrogenation. Energization of the mitochondrial membrane inhibited the initial rate of non-energy-linked reduction of DPN⁺ by TPNH. Under equilibrium conditions, membrane energization activated this reaction, especially in the presence of magnesium ions. These results are interpreted to indicate that activation of the DT-transhydrogenase rate (normally observed on addition of an energy donor) is a function separate from the increase in the apparent equilibrium constant of the reaction.

Mitochondrial non-energy-dependent pyridine nucleotide transhydrogenase which catalyzes the reversible stereospecific transfer of a hydride ion equivalent from the 4A side of the nicotinamide ring of DPNH to the 4B side of TPNH (Lee *et al.*, 1965) (eq 1) was first described in beef heart mitochondria by Kaplan and coworkers (Kaplan *et al.*, 1953). Subsequently, the membrane-bound enzyme has been detected in a number of other types of mitochondria (Kaplan *et al.*, 1953) and several genera of bacteria (Keister and Yike, 1967; Asano *et al.*, 1967; Orlando, 1968; Bragg and Hou, 1968).



In submitochondrial particles the initial rate of the backward reaction is several fold greater than the forward rate. However, energization of the membrane oxidative phosphorylation system by the oxidation of respiratory substrates or ATP hydrolysis increases the rate of TPN⁺ reduction 4- to 6-fold and the apparent equilibrium constant¹ under proper conditions is increased from about unity to nearly 500 (Lee and Ernster, 1964). The observation that this energy-dependent reaction is inhibited by uncouplers and inhibitors of oxidative phosphorylation led to the proposal that a nonphosphorylated high-energy intermediate of oxidative phosphorylation (X~Y) participates catalytically during hydrogen transfer in the en-



ergy-linked transhydrogenase (eq 2) (Ernster and Lee, 1964).

Treatment of bovine heart mitochondria with detergents results in the solubilization of pyridine nucleotide transhydrogenase activity (Kaufman and Kaplan, 1961). Several lines of evidence indicate that the solubilized enzyme functions in the catalysis of the energy-linked and non-energy-linked reactions, (a) an antibody prepared against the solubilized enzyme inhibits both transhydrogenase reactions catalyzed by submitochondrial particles (Kawasaki *et al.*, 1964), (b) both reactions are 4A specific with regard to DPNH and 4B specific with regard to TPNH (Kawasaki *et al.*, 1964; Lee *et al.*, 1965), and (c) a protein factor has been isolated from *Rhodospirillum rubrum* chromatophores which is required for the reconstitution of both reactions with factor-depleted membranes (Fisher and Guillory, 1971).

Until recently, the energy-linked reaction was considered to be irreversible. However, Skulachev and coworkers (Grinius *et al.*, 1970; Isaev *et al.*, 1970) showed that the oxidation of TPNH by DPN⁺ catalyzed by submitochondrial particles could be coupled to the transport of lipid soluble anions into the particles, a function also observed on energization of the membrane by addition of ATP or succinate. Further evidence that the transhydrogenase is in reversible equilibrium with the energy-conservation system of the membrane was provided by Van de Stadt *et al.* (1971). These workers showed that the backward reaction of eq 2 resulted in induction of energy-dependent enhancement of ANS² fluorescence and a red shift

[†] From the Department of Chemistry, University of California at San Diego, La Jolla, California 92037. Received October 25, 1972. This work was supported in part by grants from the National Institutes of Health (CA-11683) and the American Cancer Society (P-77M). R. R. F. was a Postdoctoral Trainee of the U. S. Public Health Service.

[‡] Present address: Department of Chemistry, University of South Carolina, Columbia, S. C. 29208.

¹ The apparent equilibrium constant for the energy-linked transhydrogenase reaction is underestimated to the extent that the non-energy-linked reaction is operative under the assay conditions (Rydström *et al.*, 1970).

² Abbreviations used are: ANS, 8-anilino-naphthalene-1-sulfonic acid; TN-TPN⁺, oxidized thionicotinamide adenine dinucleotide phosphate; AcPy-DPN⁺, 3-acetylpyridine-DPN⁺; TD-transhydrogenase, the reduction of DPN⁺ by TPNH; DT-transhydrogenase, the reduction of TPN⁺ by DPNH.

in the spectrum of cytochrome *b*, and that it could be coupled to the synthesis of ATP from ADP and phosphate.

The mode of energy input to the transhydrogenase system is unknown. Three mechanisms are presently being considered, (a) DPNH or TPN⁺ reacts chemically with X~Y to form an energized pyridine nucleotide which is in turn deenergized as a result of hydrogen transfer (Ernster and Lee, 1964); (b) energization of the membrane converts the transhydrogenase from an inactive to an active conformation, *i.e.*, it has been proposed that the active conformation reverts to its inactive form during each turnover (Rydström *et al.*, 1970, 1971); and (c) the transhydrogenase couples the transport of protons across the membrane to hydrogen transfer between the two substrates (Mitchell, 1966; Skulachev, 1970).

In this paper we report on the influence of membrane energization on the initial and equilibrium kinetics, as well as the kinetic isotope effects of beef heart mitochondrial pyridine nucleotide transhydrogenase reactions.

Materials and Methods

Frozen beef heart mitochondria (Ziegler and Doeg, 1962), a gift of Dr. Y. Hatefi, was used as starting material for the preparation of phosphorylating submitochondrial particles (Löw and Vallin, 1963). The particles were suspended in 0.25 M sucrose at a concentration of 60 mg/ml and stored at -65°.

ANS, a gift of Dr. R. Guillory and a product of Eastman Company, was recrystallized three times from water. The extinction coefficient (ϵ) was taken to be $4.95 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Weber and Young, 1964).

TN-TPN⁺ was synthesized by the method of Walter and Rubin (1963). Lactate dehydrogenase from chick heart was prepared as previously described (Pesce *et al.*, 1964) and dialyzed free of ammonium sulfate prior to use.

Chemical sources were: [4-³H]DPN⁺ of 99% chromatographic purity, New England Nuclear; 99.8 atom % deuterated ethanol and 99.7 atom % deuterium oxide, Merck, Sharp and Dohme; ATP, DPN⁺, DPNH, 3-acetylpyridine-DPN⁺, TPN⁺, and TPNH, P-L Laboratories; Torula yeast glucose-6-phosphate dehydrogenase, glucose 6-phosphate, salt-free yeast alcohol dehydrogenase, rotenone, oligomycin, and reagent grade Trizma base, Sigma Chemical Co.; sodium succinate and sodium pyruvate, Calbiochem; α -ketoglutarate, Mann Research Laboratories; 2,5-diphenyloxazole and *p*-bis[2-(5-phenyloxazolyl)]-1-benzene, Pilot Chemicals; and glutamate dehydrogenase, Boehringer Mannheim Corp.

Stopped-Flow Experiments. Initial rates of transhydrogenation were determined with an Aminco-Morrow stopped-flow apparatus equipped with an Aminco grating monochromator and a reaction cell with a 1-cm light path. The absorbance increase at 400 m μ which results from the reduction of TN-TPN⁺ by DPNH was recorded on a strip recorder. The rate of the energy-dependent transhydrogenase was replotted after subtracting the non-energy-dependent reaction rate, which was determined in a separate experiment under identical conditions in the absence of succinate or ATP. Both syringes contained the same concentrations of buffer and sucrose in order to maintain iso-osmolality during mixing, and to minimize light-scattering effects.

The rate of energy-dependent ANS fluorescence enhancement (Azzi *et al.*, 1969) was determined on the same instrument equipped with a fluorescence attachment. The fluorescence excitation wavelength was 380 m μ . Emitted light was filtered through a Corning CS 3-73 filter.

Experiments with D₂O. Transhydrogenase reactions and ANS fluorescence enhancement studies were carried out where indicated, in 98.7 atom % of deuterium oxide. The pD of the solutions was calculated according to the equation: pD = recorded pH + 0.4 (Glasoe and Long, 1960).

Determination of [4-³H]DPN⁺-H₂O Tritium Exchange. The exchange reaction was initiated by addition of [4-³H]-DPN⁺ to submitochondrial particles which were preincubated for at least 3 min with rotenone in a reaction medium. The description of the media is given in the descriptions of the individual experiments. At indicated times, a 1-ml aliquot was removed, pipetted into 0.2 ml of 1 N NaOH, and used for the analysis of DPNH and TPNH. Within 5 sec, a 0.5-ml aliquot was removed, and transhydrogenation reaction was terminated by heating in a boiling-water bath for 1 min, and then used for analysis of tritium exchange from [4-³H]DPN⁺ to water.

After neutralization of the first aliquot with 0.2 ml of 1 N acetic acid, and centrifugation to remove precipitated membranes, the DPNH concentration was determined from the decrease in absorbance at 340 m μ , assuming an ϵ of $6.22 \text{ mm}^{-1} \text{ cm}^{-1}$, on addition of 2 mM pyruvate and 10 μg of lactate dehydrogenase. TPNH was then determined in the same aliquot by its oxidation on addition of 5 mM NH₄Cl, 14 mM α -ketoglutarate, and 50 μg of glutamate dehydrogenase.

Medium water of heat-treated aliquots was isolated by lyophilization (Lee *et al.*, 1965) and 0.2-ml aliquots were added to 10 ml of scintillation solution containing 5 g of 2,5-diphenyloxazole, 0.2 g of *p*-bis[2-(5-phenyloxazolyl)]-benzene, and 100 g of naphthalene in 1 l. of Scintillation Grade dioxane. Rates of exchange were corrected for the tritium found in the reaction medium water (10–15 cpm above background) at zero time. Radioactivity was determined with a Beckman LS-100 liquid scintillation counter using the full-spectrum tritium window.

Results

Stopped-Flow Studies on the Pyridine Nucleotide Transhydrogenase. DPNH and TPNH have identical absorption spectra. Therefore, the direct spectrophotometric determination of the kinetics of TPN⁺ reduction by DPNH generally is accomplished by a continuous enzymatic regeneration either of the DPNH or TPN⁺ during the course of the assay (Ernster and Lee, 1967). However, dependence on a regenerating system precludes a clear-cut determination of the initial reaction kinetics. For example, we observed time lags in the energy-linked and non-energy-linked transhydrogenase reactions, which were decreased in length upon increasing the alcohol dehydrogenase concentration of the DPNH regenerating system. Other laboratories have also reported lags in the energy-linked reaction, but they have not determined whether the lag resulted from an inadequate regenerating system or if the initial rate of energy input to the transhydrogenase was rate limiting, *i.e.*, the formation or utilization of the nonphosphorylated high-energy intermediate of the oxidative phosphorylation system (Asano *et al.*, 1967; Lee *et al.*, 1964).

Kawasaki *et al.* (1964) reported that the submitochondrial particle enzyme catalyzes the reduction of the 3-acetylpyridine and thionicotinamide analogs of TPN⁺ by DPNH and that due to the differences in the absorption spectra of the reduced TPN⁺ analogs from DPNH, the reaction may be assayed in the absence of regenerating systems. This suggested that valid initial rates of transhydrogenation could be obtained using

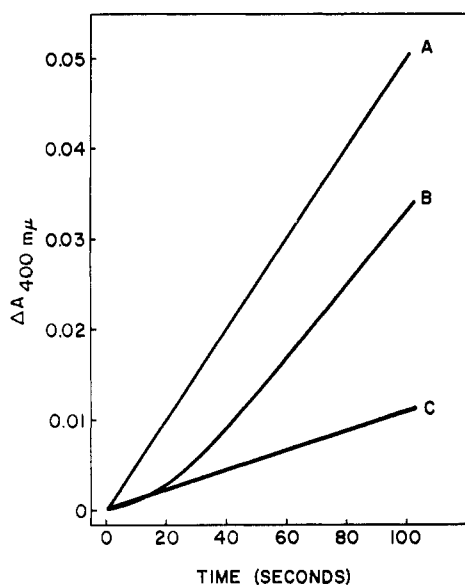


FIGURE 1: Stopped-flow time course study of the energy-linked transhydrogenase reaction. Experiment A: submitochondrial particles (0.6 mg of protein) were preincubated for 2 min in syringe 1 (2 ml) containing 50 mM Tris-HCl buffer (pH 7.6), 175 mM sucrose, 10 μ g of rotenone, 0.14 mM TN-TPN⁺, 50 mM MgSO₄, and 10 mM sodium succinate. Syringe 2 contained 50 mM Tris-HCl buffer, 175 mM sucrose, and 0.18 mM DPNH. Experiment B: submitochondrial particles were preincubated for 2 min in syringe 1 containing 50 mM Tris-HCl buffer, 175 mM sucrose, 10 μ g of rotenone, 0.14 mM TN-TPN⁺, and 50 mM MgSO₄. Syringe 2 contained 50 mM Tris-HCl buffer, 175 mM sucrose, 0.18 mM DPNH, and 10 mM sodium succinate. Experiment C (non-energy-linked reaction) was performed as experiment B except sodium succinate was omitted from syringe 2.

an analog of TPN⁺. TN-TPN⁺ was the analog of choice, since it gives a maximum velocity of about twice that of the 3-acetylpyridine analog (Kawasaki *et al.*, 1964) and the extinction coefficient of the former at 395 m μ is about 11.3 mm⁻¹ cm⁻¹, compared to 5.1 mm⁻¹ cm⁻¹ for the latter assayed at 375 m μ (Stein *et al.*, 1959).

A comparison of the rates of non-energy-linked and energy-linked transhydrogenation at saturating concentrations of TPN⁺ and TN-TPN⁺ is given in Table I. It can be seen that the maximum velocity of TPN⁺ reduction by DPNH is about three times that of TN-TPN⁺. However, the ratios of the rates of the two reactions were similar for both hydrogen acceptors. Consistent with previous reports (Hommes, 1963; Lee and Ernster, 1964), magnesium ions were found to inhibit the non-energy-linked transhydrogenase to approximately the same extent and to have little influence on the energy-linked reaction when TN-TPN⁺ replaced TPN⁺. The optimal pH for TN-TPN⁺ as well as TPN⁺ reduction in the energy-linked reaction was 7.4. These results suggest that TN-TPN⁺ is a valid substitute for TPN⁺ in the study of the initial kinetics of hydrogen transfer.

Figure 1 shows the time course of TN-TPN⁺ reduction by DPNH catalyzed by beef heart submitochondrial particles. In expt B, energy-linked transhydrogenation was initiated by the addition of DPNH and succinate to particles preincubated in the presence of TN-TPN⁺. A lag of about 30 sec was observed before transhydrogenation proceeded at its maximum rate. A lag of similar duration was recorded when ATP was substituted for succinate as an energy donor. By preincubating the submitochondrial particles with succinate (or ATP) and

TABLE I: Comparison of the Rates of TPN⁺ and TN-TPN⁺ Reduction by DPNH.^a

Additions	Hydrogen Acceptor	Trans-hydrogenase Rate ^b	EL:NEL ^c
None	TPN ⁺	17.3	
10 mM Succinate	TPN ⁺	46.8	2.7
None	TN-TPN ⁺	6.4	
10 mM Succinate	TN-TPN ⁺	16.2	2.5
20 mM MgSO ₄	TPN ⁺	7.3	
20 mM MgSO ₄ -	TPN ⁺	41.1	5.6
10 mM succinate			
20 mM MgSO ₄	TN-TPN ⁺	3.4	
20 mM MgSO ₄ -	TN-TPN ⁺	15.8	4.7
10 mM succinate			

^a The reaction medium at 22° contained: 75 mM Tris-HCl buffer (pH 7.4), 190 mM sucrose, 0.15 mM DPNH, 0.28 mM TPN⁺ or TN-TPN⁺, 64 units of yeast alcohol dehydrogenase, 200 mM ethanol, 2 μ g of rotenone, and 0.29 mg of submitochondrial particle protein in a final volume of 1 ml. The reduction of TPN⁺ was followed by the increase in absorbance at 340 m μ assuming a millimolar extinction coefficient of 6.22 and the reduction of TN-TPN⁺ was followed at 395 m μ assuming an extinction coefficient of 11.3. ^b nmol of TPN⁺ or TN-TPN⁺ reduced min⁻¹ mg⁻¹ particle protein. ^c The ratio of the rates of the energy-linked (EL) to the non-energy-linked transhydrogenase (NEL) reactions.

TN-TPN⁺ before adding DPNH, it was shown that this lag is the result of a rate-limiting input of energy to the transhydrogenase system. In this case (expt A) no lag was observed at the onset of transhydrogenation. The same result was obtained on addition of both DPNH and TN-TPN⁺ to particles preincubated with the energy donor. In addition, no lag was observed in the initial rate of non-energy-linked TN-TPN⁺ reduction when DPNH was added to initiate the reaction in the absence of the energy donor (expt C). No change was seen in the light scattering of the submitochondrial particle suspension on addition of succinate or ATP in the absence of pyridine nucleotides.

Influence of D₂O on Transhydrogenation. Succinate oxidation by submitochondrial particles is decreased from 40 to 50% by D₂O, although the rate of ATP synthesis from ADP and phosphate coupled to electron transport is only slightly decreased (Margolis *et al.*, 1966; Murasha and Slater, 1966). Thus P:O ratios are significantly increased in D₂O compared to those measured in H₂O. Margolis *et al.* (1966) reported that D₂O also inhibits the energy-linked, but not the non-energy-linked transhydrogenase at pH(D) 7.8. Furthermore, inhibition of succinate oxidation by malonate in H₂O to levels as seen in D₂O in the absence of malonate did not appreciably decrease the rate of energy-linked TPN⁺ reduction. These workers suggested that this inhibition by D₂O is at the level of utilization and not at the level of formation of some non-phosphorylated high-energy intermediate of the energy conservation system.

Figure 2 illustrates the comparison of the effect of various pH and pD values on the energy-linked and non-energy-linked reduction of TN-TPN⁺ by DPNH. The pD optimum for the energy-linked reaction is shifted about 0.4 of a unit

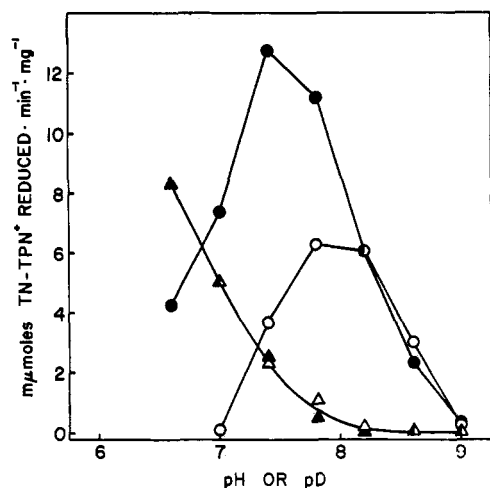


FIGURE 2: Influence of pH and pD on the rate of pyridine nucleotide transhydrogenase. Non-energy-linked transhydrogenase in H_2O (\blacktriangle), in 98.7% D_2O (\triangle), energy-linked transhydrogenase in H_2O (\bullet), in 98.7% D_2O (\circ). Reactions were assayed at 22° in the presence of 20 mM MgSO_4 as described in Table I. 3 mM sodium succinate was the energy source. The rate of the energy-linked reaction represented is the rate of TN-TPN $^+$ reduction in the presence of succinate less the rate of reduction in the absence of succinate.

higher from its optimal pH of 7.4. The V_{max} for the reaction in D_2O was 50% of that in H_2O . Interestingly, at pD values below 7.0 the energy-linked reaction was completely inhibited, although at pH 7.0 the rate of TN-TPN $^+$ reduction was nearly 50% maximal. In D_2O half-maximal rate of transhydrogenation was found at pD 7.4. At pD and pH values greater than 8.4, transhydrogenation proceeded at nearly identical rates in both D_2O and H_2O .

The influence of D_2O on the initial rate of energy-linked transhydrogenation is demonstrated in Figure 3. Consistent with the data presented in Figure 2, the rate of hydrogen transfer in D_2O at pD 7.4 was about 35% of the rate in H_2O . Significantly, the initial lag time was increased from 35 to 40 sec, in H_2O , to between 55 and 60 sec, in D_2O . There was no energy-linked transhydrogenation measured in D_2O during the first 30 sec although there was no decrease in the rate or a lag in the non-energy-linked reaction. In other D_2O experiments, total initial inhibition of transhydrogenation was observed from about 5 to 30 sec, but in all cases the length of the lag before achieving maximal reaction rates was similar to those given above. The differences of the "dead time" in the lag may be related to the efficiency of energy coupling, in the various submitochondrial particle preparations studied.

The initial rates of the transhydrogenase reactions are informative, in that they provide a means to investigate the rate of energization of the membrane to a state useful in promoting energy-linked reactions. A number of fluorescent probes have been used previously to study the time course of mitochondrial membrane structural changes (Azzi *et al.*, 1969; Chance, 1970; Datta and Penefsky, 1970; Radda, 1971). One such probe, 8-anilidonaphthalene-1-sulfonate, becomes fluorescent on being bound to the deenergized submitochondrial membrane. The fluorescence of ANS is further enhanced upon energization of the membrane oxidative phosphorylation system, by the addition of respiratory substrate or ATP (Azzi *et al.*, 1969; Datta and Penefsky, 1970). Chance (1970) has concluded that the configuration of the energized state of the membrane is achieved only after steady-state electron transport has been

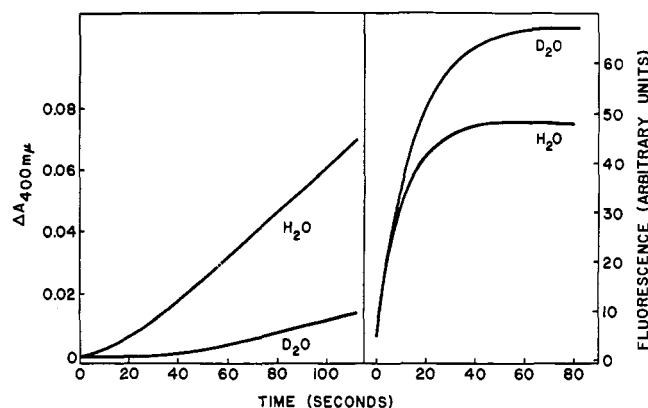


FIGURE 3: Influence of D_2O on the energy-linked transhydrogenase lag and ANS fluorescence enhancement. (A) The stop-flow transhydrogenase medium contained in syringe 1 (2 ml) in H_2O or 98.7 atom % D_2O , 50 mM Tris-HCl buffer (pH 7.4) or pD 7.4, 175 mM sucrose, 0.18 mM DPNH, 0.28 mM TN-TPN $^+$, 0.6 mg of submitochondrial particle protein, 10 mM MgSO_4 , and 10 μg of rotenone. Syringe 2 contained, in 2 ml, 50 mM Tris buffer and 10 mM sodium succinate. Curves were obtained by subtracting rates recorded in the absence of succinate from those in the presence of succinate. (B) ANS fluorescence enhancement experiments were carried out in H_2O or 98.7 atom % of D_2O . Syringe 1 contained in 2 ml, 50 mM Tris buffer (pH 7.4) or pD 7.4, 175 mM sucrose, 0.085 mM ANS and 10 mM MgSO_4 . Syringe 2 contained, in 2 ml, 50 mM Tris buffer, 175 mM sucrose, and 10 mM sodium succinate.

established; this conclusion was reached on the basis of kinetic studies on electron transport, and of energy-linked ANS fluorescence enhancement. It was, therefore, of interest to compare the rate of membrane activation upon the addition of high-energy donor measured by the transhydrogenase lag and by ANS fluorescence enhancement in H_2O and D_2O . As shown in Figure 3, the extent of ANS fluorescence enhancement in D_2O upon addition of succinate to submitochondrial particles preincubated with ANS was 1.4 times of that in H_2O (Radda, 1971). The half-times of fluorescence enhancement in D_2O and H_2O were 11 and 8 sec, respectively. Clearly, achievement of maximal rates of transhydrogenation in H_2O and D_2O coincides with the attainment of maximal ANS fluorescence. In D_2O , under proper conditions, it appears that ANS fluorescence enhancement is over 90% complete before the onset of transhydrogenation. These data may be interpreted to indicate that the rate of energization of the transhydrogenase system is slower than the rate of the energy-linked process leading to the fluorescence enhancement of ANS.

Effect of DPNH Deuteration on the Rate on the Energy-Linked and Non-Energy-Linked Transhydrogenase Reactions. Studies on the membrane-bound pyridine nucleotide transhydrogenase of mitochondria and certain bacteria have demonstrated that the reaction involves a stereospecific transfer of a hydride ion equivalent from the 4A side of DPNH to the 4B side of TPNH (Lee *et al.*, 1965; Orlando, 1968; Fisher and Guillory, 1971). In an effort to ascertain whether the probable rate-determining step of the transhydrogenase reactions is C-H bond cleavage in DPNH, the effect on the reaction of substituting a deuterium atom for hydrogen at the 4A locus of DPNH was investigated. From Table II it can be seen that no kinetic isotope effect was observed when [4A- ^3H]DPND was substituted for DPNH in either the energy-linked or non-energy-linked reduction of TPN $^+$ or TN-TPN $^+$. This result indicates that the rate deter-

TABLE II: Influence of Deuteration of DPNH on the Rate of Energy-Linked and Non-Energy-Linked Transhydrogenase Reactions.^a

Hydrogen Donor	Hydrogen Acceptor	Transhydrogenase Rate	
		nmol of TPN ⁺ or TN-TPN ⁺ reduced min ⁻¹ mg ⁻¹	
		- ATP	+ ATP
Experiment 1			
DPNH	TPN ⁺	23	44
[4A- ² H]DPND	TPN ⁺	21	42
Experiment 2			
DPNH	TN-TPN ⁺	5	14
[4A- ² H]DPND	TN-TPN ⁺	5	13

^a The reaction medium at 23° contained 75 mM Tris-HCl buffer (pH 7.4), 190 mM sucrose, 64 units of yeast alcohol dehydrogenase 0.28 mM TPN⁺ or TN-TPN⁺, 0.15 mM DPN⁺, and 5 mM potassium cyanide. 5 mM ATP was added with 20 mM MgSO₄. Unlabeled DPNH and [4A-²H]DPND were obtained on reduction of DPN⁺ by addition of 300 mM ethanol and fully deuterated ethanol, respectively. On cessation of DPN⁺ reduction, 0.29 mg of submitochondrial particle protein was added to initiate the transhydrogenase reaction.

minating step in transhydrogenation is not C-H bond cleavage, but is consistent with steady-state kinetic data, indicating a Theorell-Chance mechanism involving a ternary complex with a short lifetime (Rydström *et al.*, 1971). However, the fact that D₂O inhibits the maximum velocity of the energy-

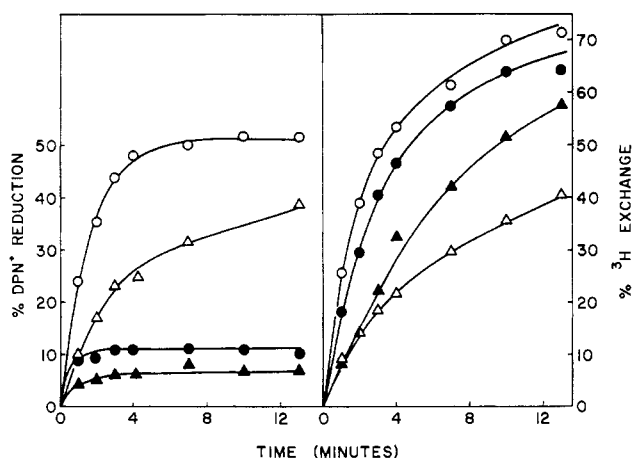
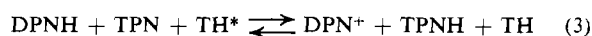


FIGURE 4: Effect of succinate on the [4-³H]DPN⁺-H₂O exchange in the presence and absence of magnesium sulfate. TD-transhydrogenase in the absence of MgSO₄ and succinate (○), in the absence of MgSO₄ plus succinate (●), plus MgSO₄ in the absence of succinate (Δ), plus MgSO₄ plus succinate (▲). The reaction mixture at 30° contained, in 15 ml, 100 mM Tris-acetate buffer (pH 8.0), 0.1 mM [4-³H]DPN⁺ (7 × 10⁴ cpm), 0.11 mM TPNH, 200 μg of rotenone, 200 μg of oligomycin, and 14 mg of submitochondrial particle protein. 14.3 mM MgSO₄ and 40 mM sodium succinate were added where indicated. Oxygen was equilibrated with the reaction mixture in a 50-ml beaker by gyrotory shaking at 140 rpm.

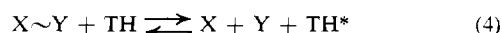
linked transhydrogenase (Figure 2) suggests that product dissociation is not limiting in this reaction.

Influence of Membrane Energization on the Rate of TD Transhydrogenation. Rydström *et al.* (1970) found by coupling the exchange of tritium from the 4B locus of DPNH to water, resulting from the reduction of [4-³H]DPN⁺ by TPNH, that the TD transhydrogenation was significantly inhibited during oxidation of succinate. This was carried out under conditions when rotenone inhibited membrane-bound DPNH-dehydrogenase. Other related studies prompted these workers to propose that energization of the membrane converts an inactive conformation of the transhydrogenase (TH) to an active conformation (TH*).

It was postulated (Rydström *et al.*, 1970, 1971) that the relative concentration of TH and TH* is determined by the ratios of the concentrations of pyridine dinucleotides (eq 3)



and by the energy level of the oxidative phosphorylation coupling system (eq 4). The diminution in the rate of DPN⁺ re-



duction in the TD hydrogen transfer would be a function of the fraction of TH converted to TH* during membrane energization. Further, as predicted from eq 3 and 4, Rydström *et al.* (1971) showed DPN⁺ and TPNH to be competitive with X~Y in promoting the proposed conversion of the inactive transhydrogenase to the active transhydrogenase.

As shown in Figure 4, the data of Rydström *et al.* (1970) were confirmed, in that in the absence of magnesium ion, the initial rate of tritium exchange to water from [4B-³H]DPNH, formed on reduction of [4-³H]DPN⁺ by TPNH, was more rapid in the nonrespiring particles than in the respiring particles. Due to the higher apparent equilibrium constant of the energy-linked reaction there was less DPNH present in the reaction mixture at any given time under energy-linked than under non-energy-linked conditions. Thus, it was possible that the differences in the rate of exchange could be a result of a limiting concentration of the membrane-bound DPNH-dehydrogenase (Lee *et al.*, 1965). It was shown that this was not the case since the addition to the [4B-³H]DPNH-H₂O exchange medium of sufficient pig heart lipoic dehydrogenase to catalyze the exchange of the 4B proton of DPNH to water at a rate comparable to that catalyzed by the mitochondrial particles did not stimulate the tritium exchange. It should be noted that after the first minute, the increments of tritium exchange were nearly identical in the presence or absence of respiration (Figure 4).

Addition of magnesium ions resulted in the inhibition of [4-³H]DPN⁺ reduction by TPNH as well as the rate of [4B-³H]DPNH-H₂O exchange in the presence and absence of respiration. However, the exchange was inhibited to a greater extent by magnesium ions under nonrespiring conditions than when succinate was oxidized concomitantly. This resulted in no apparent inhibition of the TD-transhydrogenase on membrane energization; in fact, after 2 min, the rate of exchange was faster during succinate oxidation (Figure 4).

These data suggested that an investigation of the rate of TD transhydrogenation at equilibrium conditions was essential. The transhydrogenase reaction was allowed to come to equilibrium both in the presence and absence of succinate and magnesium sulfate. The apparent equilibrium constants of the

TABLE III: Influence of Succinate and Magnesium Sulfate on the $[4\text{-}^3\text{H}]\text{DPN}^+ \text{--H}_2\text{O}$ Exchange at the Transhydrogenase Steady State.^a

Additions	cpm Exchanged ($\times 10^{-3}$)	% Exchange	$[\text{DPN}^+]$, μM ^b	Rel Exchange Rate ^c	K_{eq}
Complete	21.9	13.7	63	1.00	1
Complete, plus succinate	26.9	16.9	99	1.83	86
Complete, plus MgSO_4	3.2	2.0	64	0.15	1
Complete, plus succinate, plus MgSO_4	17.0	10.6	106	1.29	172

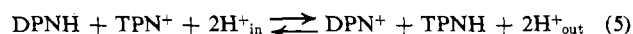
^a Initially, the reaction mixtures at 30° contained 100 mM Tris-acetate buffer (pH 8.0), 250 mM sucrose, 0.115 mM DPN^+ (unlabeled), 0.125 mM TPNH, 200 μg of rotenone, 40 μg of oligomycin, and 14 mg of submitochondrial particle protein in a final volume of 14.9 ml. When added, MgSO_4 was 13.3 mM and sodium succinate was 20 mM. The transhydrogenase reactions were allowed to come to equilibrium in the presence or absence of succinate. MgSO_4 was then added when indicated and the exchange reaction initiated 1 min later by the addition of 1.6×10^5 cpm of $[4\text{-}^3\text{H}]\text{DPN}^+$ (2 mCi/mg) in 0.1 ml. After 1 min, the amount of tritium exchange from $[4\text{-}^3\text{H}]\text{DPN}^+$ to medium water was determined as described under Materials and Methods. ^b Concentration of DPN^+ at equilibrium. ^c Since the same quantity of $[4\text{-}^3\text{H}]\text{DPN}^+$ was added to each reaction mixture containing different concentrations of DPN^+ , the relative tritium exchange rates were calculated according to the formula: $[\text{DPN}^+]_x \cdot \% \text{ exchange}_x / [\text{DPN}^+]_0 \cdot \% \text{ exchange}_0 = \text{relative exchange rate}$, where $[\text{DPN}^+]_0$ and $[\text{DPN}^+]_x$ are the steady-state concentrations of DPN^+ in the complete system and in the presence of indicated additions, respectively.

reactions in the absence of succinate were about 1, while those in the presence of succinate and succinate plus magnesium sulfate were 86 and 172, respectively. The greater apparent equilibrium constant in the presence of magnesium ions results from a lower contribution of the non-energy-linked TD reaction (Rydström *et al.*, 1970). Carrier-free $[4\text{-}^3\text{H}]\text{DPN}^+$ was then added to the reaction mixture and the initial rate of tritium exchange from the pyridine nucleotide to medium water was determined. The rates of exchange were linear for at least 5 min in the presence of magnesium sulfate and for at least 2 min in its absence. As shown in Table III, the relative ratio of $[4\text{-}^3\text{H}]\text{DPNH}\text{--H}_2\text{O}$ exchange in the absence of magnesium sulfate was increased from unity to 1.83 in the presence of succinate. As under initial velocity conditions, Table I, magnesium sulfate at 13.3 mM inhibited the TD-transhydrogenase by 85% compared to the control. Succinate oxidation stimulated the magnesium sulfate inhibited exchange more than 8-fold to about 70% of that measured with succinate in the absence of magnesium sulfate.

Discussion

The initial lag observed in the energy-linked, but not during the DT non-energy-linked pyridine nucleotide transhydrogenase reaction (Figures 1 and 3) cannot be the result of a rate limiting step at the level of formation of an energized pyridine nucleotide (Ernster and Lee, 1964), for preincubation of submitochondrial particles with oxidizable substrate or ATP in the absence of either DPNH or TN-TPN⁺ eliminated the lag. These experiments are not intended to prove that a modified pyridine nucleotide synthesized in a reaction with $\text{X}\sim\text{Y}$ is not a substrate for transhydrogenation, but only that if it is formed it seems to be formed by a fast reaction. It appears as though the lag arises either from a conversion of the transhydrogenase enzyme from a less active to a more active form, or from a slow accumulation of an optimal level of $\text{X}\sim\text{Y}$ (or proton gradient as predicted by the Mitchell hypothesis (Mitchell, 1966)). Once formed $\text{X}\sim\text{Y}$ could interact with the transhydrogenase resulting in its activation through conformational alteration (Rydström *et al.*, 1970, 1971) or react

with DPNH or TPN⁺ to form an energized pyridine nucleotide (Ernster and Lee, 1964). In the case of the Mitchell (1966) mechanism, transport of protons down an electrochemical gradient from the inside of the membrane particles to the medium is coupled to the transfer of hydrogen from DPNH to TPN⁺ via a specific hydride ion carrier (eq 5).



In this regard it is of interest that the kinetics of proton accumulation by submitochondrial particles are similar to those fluorescence changes measured with ANS when the membranes are energized (Chance, 1970).

Considerable conformation changes of the inner membrane of intact mitochondria on energization have been noted (Penniston *et al.*, 1968). If the kinetics of ANS fluorescence enhancement observed on addition of energy donor represent membrane structural changes (Azzi *et al.*, 1969; Datta and Penefsky, 1970; Radda, 1971), it is clear from the experiments with D_2O (Figure 3) that the activation of the transhydrogenase system occurs subsequent to gross energization of the membrane. Azzi *et al.* (1969) reported that reversal of electron transport measured by the energy-linked reduction of DPN^+ by succinate is also preceded by the function indicated by energy-dependent ANS fluorescence enhancement.

At pH(D) 7.4 the rate of energy-linked transhydrogenation in H_2O was three times that in D_2O at saturating concentrations of DPNH and TN-TPN⁺ (Figure 2). The initial lag in TN-TPN⁺ reduction was increased from about 30 sec in H_2O to about 50 sec in D_2O (Figure 3). It was shown that this diminution in rate was not the result of incorporation of deuterium into the 4A locus of DPNH associated with a deuterium isotope effect as illustrated by the data given in Table II. In addition, Lee *et al.* (1965) have demonstrated that submitochondrial particles do not catalyze the exchange of tritium from $[4\text{-}^3\text{H}]\text{DPNH}$ to water.

The lower transhydrogenase rate in D_2O may reflect a less efficient conversion of the transhydrogenase to an activated conformation or synthesis of energized substrate. At pD 7.0 no energy-linked transhydrogenase activity was detected,

while in H₂O at pH 7.0 the rate of TN-TPN⁺ reduction was over half-maximal rate (Figure 2). Since the substrates do not possess dissociable protons in this region and the non-energy-linked reaction was unaffected by substituting D₂O for H₂O, it appears that energy-linked transhydrogenation requires a rate limiting dissociation of a proton from a group with pK_a of about 7.0 on either an activated transhydrogenase enzyme or on a component of the energy-coupling system. Most acids are 3- to 5-fold weaker in D₂O than in H₂O. In D₂O the pK_a of the activating group is shifted higher by about 0.5 unit. It may be possible that one of the two amino acid residues of proteins known to exhibit pK_a's in the region of 7, i.e., the terminal α -amino group or a histidyl residue (Dixon and Webb, 1964), may be involved in the transhydrogenation reaction.

That conformational alterations occur as the result of transhydrogenase membrane energization is still an equivocal concept. Consistent, however, with this interpretation is the finding that the binary enzyme-substrate and enzyme-product dissociation constants are altered on energization (Rydström *et al.*, 1971).

The increase in rate of the DT-transhydrogenase reaction on membrane energization is an established phenomenon. Similarly, it has been known for some time that the energy linked DT-transhydrogenase reaction is unaffected by magnesium ions while the nonenergy-linked TD reaction is inhibited under initial velocity conditions (Hommes, 1963). As shown in Table III, the non-energy-linked TD-transhydrogenase at equilibrium is affected in a like manner by membrane energization and magnesium ions. That is, the rate of DPN⁺ reduction by TPNH is enhanced by concomitant succinate oxidation. Further, the inhibition of the TD reaction by magnesium ions is relieved upon membrane energization. In consideration of these data it seems reasonable to assume that membrane energization under the appropriate conditions activates the transhydrogenase in both the DT and TD directions, possibly by involving a conformational change in the transhydrogenase enzyme.

The present observations support the concept that activation of the energy-linked transhydrogenase rate on membrane energization is a separate function from the increase in the apparent equilibrium constant of the reaction.

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