# Interactions of Reduced and Oxidized Triphosphopyridine Nucleotides with the Electron-Transport System of Bovine Heart Mitochondria†

Y. Hatefi and W. G. Hanstein‡

ABSTRACT: Submitochondrial particles from beef heart oxidize reduced triphosphopyridine nucleotide (TPNH), in the absence of added diphosphopyridine nucleotide (DPN), with P:O > 2. TPNH oxidation is inhibited by rotenone, piericidin A, amytal, antimycin A, and cyanide, but not by 2-thenoyltrifluoroacetone. Submitochondrial particles are also capable of ATP-dependent TPN reduction by succinate. This reaction is inhibited by uncouplers, oligomycin, rotenone, or piericidin A. TPNH dehydrogenase (as assayed with potassium ferricyanide as acceptor) and TPNH → DPN transhydrogenase fractionate more into complex I than into complexes II, III, and IV. Similar to submitochondrial particles, the transhydrogenase activity of complex I is inhibited by palmitoyl coenzyme A. Electron paramagnetic resonance studies at 14°K have shown that TPNH reduces iron–sulfur centers 2 and 3 + 4 in complex I, but not center 1. All four centers are reduced in complex I by DPNH. Thus, the site of TPNH dehydrogenation on the respiratory chain appears to be on the substrate side of the rotenone–piericidin block and linked to iron–sulfur centers 2 and 3 + 4. Both the TPNH oxidase and the TPNH → DPN transhydrogenase activities of submitochondrial particles have their pH optima below pH 6.5, and both decrease about 40-fold when the pH of the medium is increased from pH 6 to 9. These and other results presented in this paper suggest a relationship between the electron transport linked enzyme systems concerned with TPNH dehydrogenation and TPNH → DPN transhydrogenation.

Oxidation of TPNH by way of DPN and pyridine nucleotide transhydrogenase, which is bound to the inner membrane of mammalian mitochondria, has been studied by a number of investigators (Estabrook *et al.*, 1963; Teixeira da Cruz *et al.*, 1971; Rydström *et al.*, 1971a; Kaplan, 1972). In this process DPN accepts a hydride ion from TPNH (Kaplan, 1972), and then undergoes oxidation by the normal DPNH oxidase pathway of the respiratory chain. Direct oxidation of TPNH, *i.e.*, *without DPN intervention*, by the electron-transport system of bovine heart mitochondria has been generally assumed not to take place (Hommes, 1963; Kaplan, 1972).

In 1969, Ernster *et al.* made the interesting observation that submitochondrial particles from beef heart catalyze a slow dehydrogenation of TPNH in the presence of 2,6-dichloroindophenol or cytochrome *c* as electron acceptor. Their results differ in three important respects from our findings (see Hatefi, 1973a, and below) regarding electron transfer from TPNH to oxygen by way of the respiratory chain. Thus, in their system (a) TPNH dehydrogenation could not be linked to oxygen uptake, (b) cytochrome *c* reduction by TPNH could not be inhibited by antimycin A, and (c) the latter reaction was slightly stimulated, rather than being inhibited, by rotenone. These results indicated strongly that TPNH dehydrogenation in the system studied by Ernster *et al.* was not linked to the electron-transport system. Accordingly, they concluded that TPNH dehydrogenation "may involve a partial reaction of nicotinamide nucleotide transhydrogenase" (Ernster *et al.*, 1969).

The present communication will demonstrate, however, that submitochondrial particles (ETP and $ETP_H$)[1] prepared from bovine heart can oxidize TPNH by molecular oxygen in the absence of added DPN. Data will also be presented regarding phosphorylation accompanying TPNH oxidation, energy-dependent TPN reduction by succinate, site of TPN and TPNH interaction with the respiratory chain, and the possible relationship between pyridine nucleotide transhydrogenase and the TPNH oxidase system. Data concerning TPNH oxidation by ETP, and the effects of respiratory chain inhibitors, have been published (Hatefi, 1973a), and a preliminary report of the present studies has appeared (Hatefi, 1973b).

## Methods and Materials

Oxygen uptake was measured by a Clark-type electrode at 30°, and phosphate essentially according to Takahashi (1955). ETP, $ETP_H$, and electron-transfer complexes I, II, III, and IV were prepared according to published procedures (respectively, Lee and Ernster, 1967; Hansen and Smith, 1964; Hatefi *et al.*, 1962a; Baginsky and Hatefi, 1969; Hatefi *et al.*, 1962b; Fowler *et al.*, 1962). Complex IV was further purified essentially by the method of Wharton and Tzagoloff (1967). Sodium acetoacetate was prepared according to Davies (1943). Protein was estimated by the biuret method (Gornall *et al.*, 1949). Transhydrogenase reactions were

† From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received February 2, 1973.* Supported by U. S. Public Health Service Grants AM08126 and CA-13609 to Y. H., and GM19734 to W. G. H.

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[1] Abbreviations used are: ETP and $ETP_H$, respectively, nonphosphorylating and phosphorylating preparations of submitochondrial particles; P-CoA, palmitoyl coenzyme A; AcAc, sodium acetoacetate; GSSG, oxidized glutathione; AP-DPN and AP-TPN, 3-acetylpyridine analogs of DPN and TPN; complexes I, II, III, and IV, respectively, preparations of DPNH–ubiquinone reductase, succinate–ubiquinone reductase, reduced ubiquinone–cytochrome *c* reductase, and cytochrome *c* oxidase; Succ, succinate; Fum, fumarate; Q, ubiquinone (coenzyme Q).

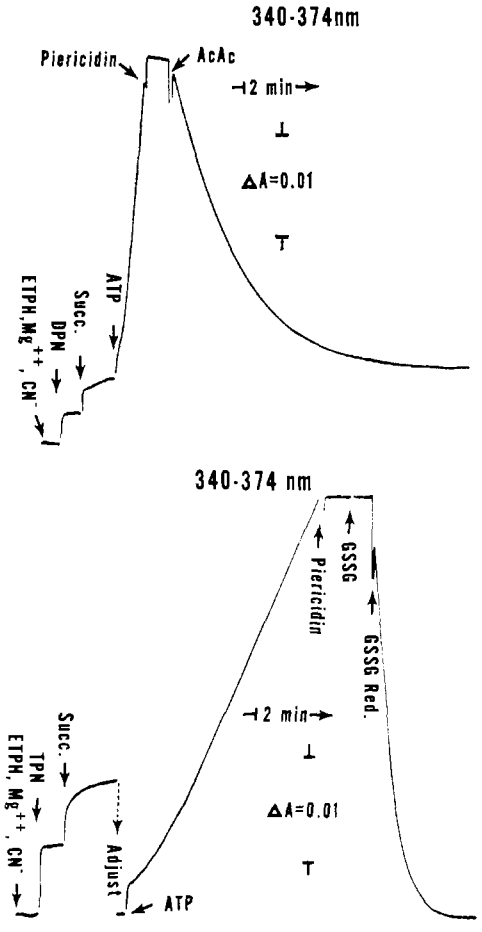


FIGURE 1: ATP-induced succinate reduction of DPN (A) and TPN (B) by $ETP_H$. Conditions: (A, top) $ETP_H$, 0.25 mg/ml; $MgCl_2$, 5 mM; NaCN, 1.5 mM; DPN, 70 $\mu$M; sodium succinate, 3.5 mM; ATP, 1.5 mM; piericidin A, 3 $\mu$M; sodium acetoacetate, 0.3 mM. (B, bottom) $ETP_H$, 0.5 mg/ml; TPN, 175 $\mu$M; GSSG, 175 $\mu$M; GSSG reductase, 1.75 $\mu$g/ml; $MgCl_2$, NaCN, ATP, and piericidin A as in part A. In this and subsequent figures, the time scales indicated are from the point of the arrows to the cross bars.

measured essentially according to Kaplan (1967) at pH 6.5 and 38° in the presence of 10 $\mu$M rotenone. A Beckman DK-2A recording spectrophotometer was used for measuring AP-DPNH or AP-TPNH production at 363 nm. The extinction coefficient used, for the difference at 363 nm for the appearance of AP-DPNH or AP-TPNH and the disappearance of DPNH or TPNH, was 5220 l. $mol^{-1} \times cm^{-1}$. Ferricyanide reductase activities were measured as before (Hatefi and Stempel, 1969), except that the medium was 0.1 M sodium phosphate (pH 6.5) containing 1 mM sodium cyanide. Energy-linked TPN and DPN reduction and TPNH reduction of electron transfer components of ETP and complex I were studied with an Aminco-Chance double-beam spectrophotometer. TPNH-reduced minus DPNH-reduced absorption spectrum of complex I was recorded with Beckman DK-2A. First-derivative electron paramagnetic resonance spectra were obtained at X band using a Jeol ME-1X spectrometer in the laboratory of Dr. Alan J. Bearden, University of California, Berkeley. Samples were frozen in liquid nitrogen and then transferred to the epr cavity; sample temperature during epr measurements was 14°K. Unless otherwise indicated, the reaction medium was 0.25 M sucrose containing 0.05 M Tris-sulfate (pH 7.5).

The possible presence of DPN–DPNH in the samples of TPNH, ETP, $ETP_H$, and complex I was checked as follows.

TABLE I: Phosphorylation Accompanying TPNH Oxidation by Submitochondrial Particles.

| Substrate | Ox Rate (nmol $min^{-1} \times mg^{-1}$ $ETP_H$ at 30°) | P:O |
|---|---|---|
| TPNH | 18–20[a] | 2.2–2.8[a] |
| 3-Hydroxybutyrate + DPN | 100 | 1.6 |
| TPNH + DPN | 100 | 1.9 |
| Succinate | 80–100[a] | 1.1–1.4[a] |

[a] Range of four separate experiments.

(1) To a reaction mixture containing oxidized glutathione and glutathione reductase was added 1.2 mM TPNH and allowed to become completely oxidized ($A_{340}$ = 0.11 OD). Then 250 $\mu$g of alcohol dehydrogenase (Sigma, type III) was added, and the absorbance change at 340 minus 375 nm was monitored upon addition of ethanol. No change occurred, even though subsequent addition of 0.25 $\mu$M DPN was clearly detected. These results showed that DPN contamination in TPNH was, if any, less than 0.02%. A similar sample of TPNH was oxidized as before and checked with acetaldehyde and alcohol dehydrogenase to see whether the remaining absorbancy of 0.11 OD at 340 nm was due to DPNH. The result was completely negative. (2) Preparations of ETP and $ETP_H$ were tested for their ability to oxidize D(−)-3-hydroxybutyrate in the absence and presence of added DPN. In the presence of sufficient amounts of added DPN, the rate of 3-hydroxybutyrate oxidation was approximately 300 nmol $min^{-1} \times mg^{-1}$ of protein at 30°. In the absence of added DPN, the rate was estimated under highly sensitive conditions and in the presence of 5.5 mg of ETP/ml, and was found to be 0.7 nmol $min^{-1}$ $mg^{-1}$ of protein. Addition of 0.18 nmol of DPN/mg of protein doubled the rate to 1.4. These results suggested that the particles contained about 0.2 nmol of DPN/mg of protein, which is in excellent agreement with the chemical determinations of Mansurova *et al.* (1972). When 0.2 nmol of DPN/mg of protein was added to a system oxidizing saturating amounts of TPNH at pH 7.5, the oxidation rate was increased by only 8%. This value agrees with the ETP-catalyzed transhydrogenase $V_{max}$ and the $K_m^{DPN}$ of 30 $\mu$M as determined by Teixeira da Cruz *et al.* (1971) for TPNH → DPN transhydrogenation. (3) The bleaching at 475 minus 510 nm of complex I (5.3 mg of protein) treated with rotenone and ethanol was studied as in Figure 3 upon sequential additions of 250 $\mu$g of alcohol dehydrogenase (Sigma, type III), 1.2 mM TPNH, and 320 $\mu$M DPNH. Alcohol dehydrogenase caused 4% bleaching, TPNH caused 56% and DPNH 40%. In this system, which lacked cytochromes and contained more than adequate amounts of rotenone, the presence of only 0.05 nmol of DPN/mg of protein could be clearly detected after addition of alcohol dehydrogenase by a slow bleaching at 475 minus 510 nm. Therefore, the absence of substantial bleaching after addition of alcohol and alcohol dehydrogenase, the partial bleaching after addition of TPNH, followed by additional bleaching after addition of DPNH, indicated that complex I could not have contained, or generated from TPNH, even 0.05 nmol of DPN/mg of protein.

Palmitoyl coenzyme A and oxidized glutathione were purchased from Sigma Chemical Co., pyridine nucleotides from P-L Biochemicals, and glutathione reductase (100 EU/mg of protein) from Calbiochem.

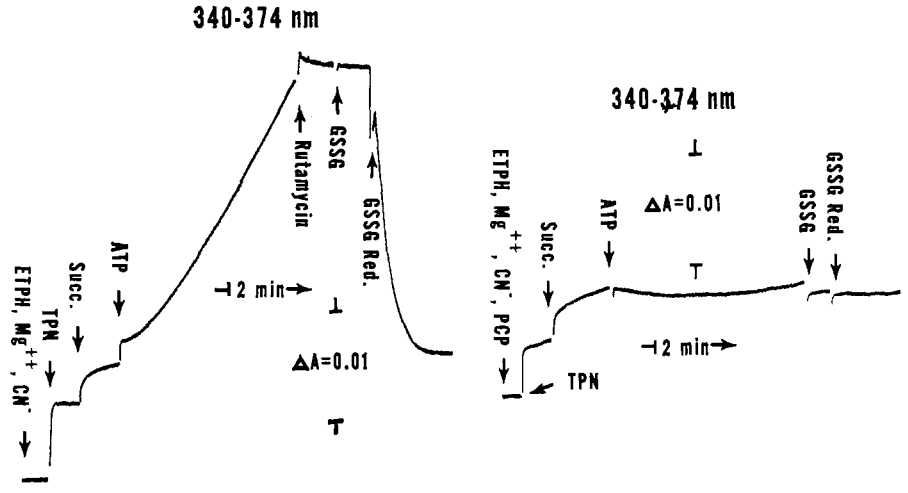


FIGURE 2: Inhibition of ATP-induced succinate reduction of TPN by rutamycin (A) and pentachlorophenol (B). Conditions were the same as in Figure 1B, except for additions of 2.8 $\mu$g/ml of rutamycin in part A and 20 $\mu$M pentachlorophenol (PCP) in part B.

## Results

*Oxidative Phosphorylation.* It was shown elsewhere (Hatefi, 1973a) that ETP preparations catalyze the rotenone- and piericidin-sensitive oxidation of TPNH at a rate of about 50 nmol $min^{-1}$ $mg^{-1}$ of protein at 30° and pH 7.5. The comparable rates, in the absence of added cytochrome *c*, for DPNH, TPNH + DPN, 3-hydroxybutyrate + DPN, and 3-hydroxybutyrate alone were, respectively, 1040, 310, 280, and 0 (Hatefi, 1973a) (also, see Methods). The oxidation rates and P:O ratios of TPNH, TPNH + DPN, 3-hydroxybutyrate + DPN, and succinate, using the Hansen–Smith-type $ETP_H$ preparation, are shown in Table I. It is seen that substrate oxidation rates are lower than ETP-catalyzed rates with the Hansen–Smith-type $ETP_H$, which is prepared in the presence of both $Mg^{2+}$ and $Mn^{2+}$ ions. However, the P:O ratios were in the expected range, and the highest values were always obtained with TPNH. The higher P:O ratios with TPNH might be due, in part, to the slower oxidation rate of this substrate. They might also be hinting at a fourth coupling site, as has been suggested by the work of Skulachev (1971) and Van de Stadt *et al.* (1971) on energy production during TPNH → DPN transhydrogenation. Experiments designed to test the possible existence of a fourth coupling site associated with TPNH oxidation are currently in progress.

*Energy-Dependent Succinate Reduction of TPN.* Figure 1 shows ATP-induced succinate reduction of DPN (Figure 1A) and TPN (Figure 1B) by cyanide-treated $ETP_H$. It is seen that both reactions require ATP and are inhibited by piericidin A (or rotenone, not shown). TPNH production was also inhibited by rutamycin (Figure 2A) and abolished when the particles were treated with the uncoupler, pentachlorophenol (Figure 2B). In the experiments of Figure 1, the rates of DPNH and TPNH production were, respectively, 70 and 5 nmol $min^{-1}$ $mg^{-1}$ of $ETP_H$ protein at 30° and pH 7.5. These results agree with the reverse electron-transfer activity data of Löw and Vallin (1963) for $ETP_H$. That the increase at 340 minus 374 nm in Figure 1A was indeed due to DPNH production was checked by adding acetoacetate to the reaction mixture, which resulted in DPNH utilization for 3-hydroxybutyrate synthesis by the particle-bound 3-hydroxybutyrate dehydrogenase. The formation of TPNH in the experiments of Figure 1B and 2 was checked with the addition of oxidized glutathione and glutathione reductase as shown.

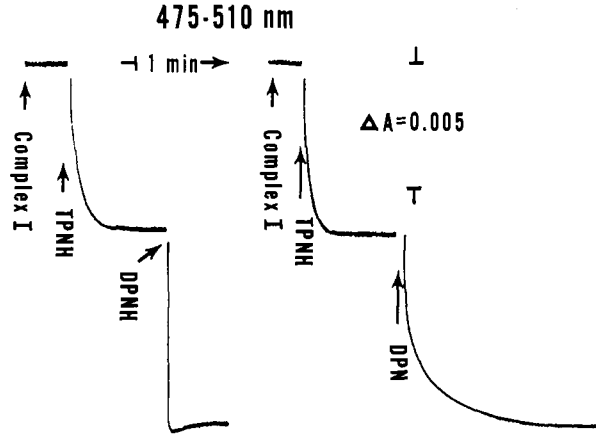


FIGURE 3: Reduction of complex I chromophores at 475 minus 510 nm by TPNH, DPNH, and TPNH + DPN. Conditions: complex I, 1.53 mg/ml in the presence of 0.1% (v/v) Triton X-100; TPNH, 175 $\mu$M; DPNH, 60 $\mu$M; DPN, 140 $\mu$M.

*Site of TPNH Interaction with the Respiratory Chain.* Rotenone- and piericidin-sensitive reduction by TPNH of cytochromes $aa_3$, $c + c_1$, and *b*, as well as the antimycin block between *b*- and *c*-type cytochromes, has been demonstrated in ETP particles (Hatefi, 1973a). It has also been shown that TPNH reduces, in a rotenone–piericidin-insensitive reaction, nearly half of the DPNH-reducible chromophores (essentially flavoproteins and iron–sulfur proteins) at 475 minus 510 nm (Hatefi, 1973a). These results suggested that TPNH interacts with the complex I region of the respiratory chain on the substrate side of the rotenone–piericidin block, and at a site near to, but not identical with, that of DPNH. In agreement with this conclusion, it was found that, when added to preparations of the individual complexes, TPNH reduced appropriate electron carriers in complex I,[2] but not in complexes II, III, and IV. The extent of complex I reduction by TPNH at 475 minus 510 nm was similar to that shown previously with ETP. Thus, as in ETP, TPNH caused nearly half as much bleaching in complex I at 475 minus 510 nm as did DPNH alone or TPNH followed by DPN (Figure 3). The nature of the chromophores reduced in complex I by

[2] It has been shown also that complex I preparations catalyze a rotenone-sensitive reduction of added ubiquinone by TPNH.

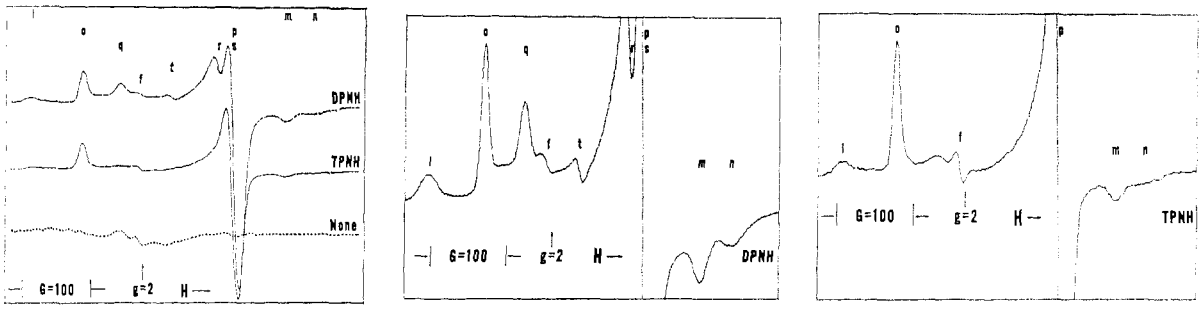


FIGURE 4: (A, left) First-derivative epr spectra of complex I treated with DPNH or TPNH. Conditions: complex I, 45 mg of protein/ml; temperature, 14°K; microwave frequency, 9.225 GHz; power, 2 mW; modulation amplitude, 6.3 G; gain, 50; $g = 2$ was at 3295 G. Where indicated 1.5 mM DPNH or TPNH was added. (B, middle; C, right) Respectively, the epr spectra of DPNH- and TPNH-treated samples of part A at a gain of 200 and 0.3-mW power.

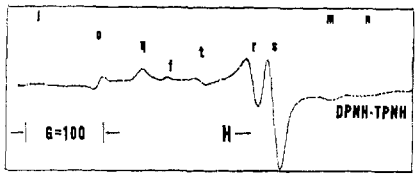


FIGURE 5: Computer-derived difference of DPNH-treated minus TPNH-treated complex I spectra shown in Figure 4.

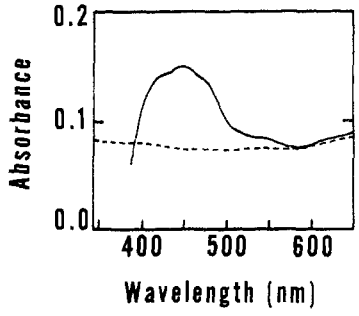


FIGURE 6: Absorption spectrum of TPNH-treated minus DPNH-treated complex I. Conditions: complex I, 6 mg of protein/ml of 0.66 M sucrose containing 50 mM Tris-chloride (pH 8.0), 1 mM histidine, and 0.25% (v/v) Triton X-100. The sample cuvet was treated with 200 μM TPNH, and the reference cuvet with 100 μM DPNH. Dashed line, untreated complex I in both cuvets.

DPNH and TPNH was further investigated by epr and light absorption spectroscopy. Low-temperature (14°K) epr spectra obtained upon addition of DPNH or TPNH to complex I are shown in Figure 4. In agreement with previous work (Orme-Johnson *et al.*, 1971), DPNH caused the reduction of iron–sulfur centers 1 (q, r, s), 2 (o, p), and 3 + 4 (l, m, n).[3] Epr absorption at "f" denotes a small amount of flavine free radical, which is also present in the control, *i.e.*, substrate-untreated sample of complex I. The TPNH-treated complex I shows comparable reduction of center 2, *partial* reduction of centers 3 + 4, but no reduction of center 1. The computer-subtracted difference between the epr spectra of the DPNH-treated and the TPNH-treated complex I is shown in Figure 5. This difference spectrum shows signals due to centers 1 and 3 + 4. It also shows a signal at $g = 2.06$, suggesting that the DPNH-induced signal marked "o" may not be due to a single component. The signal marked "t" with a $g$ value of 1.98, which appears along with center 1 reduction in these experiments, has not been further investigated. The difference in the visible light absorption of TPNH-treated and DPNH-treated complex I is shown in Figure 6. It is seen that this difference spectrum is highly suggestive of oxidized flavine. Thus, in agreement with the properties and composition of DPNH dehydrogenase previously isolated from complex I (Hatefi and Stempel, 1969), and in accord with kinetic, titration, and redox potential studies concerning iron–sulfur centers 1, 2 and 3 + 4 (Orme-Johnson *et al.*, 1971; Ohnishi *et al.*, 1972), it appears that the DPNH dehydrogenase component of complex I, which has been shown to be an iron–sulfur flavoprotein (Hatefi and Stempel, 1967), is not reducible by TPNH. These results further suggest that the site of interaction of TPNH with the respiratory chain is at the level of iron–sulfur centers 2 and 3 + 4.

*Relationship between TPNH Oxidase and Pyridine Nucleotide Transhydrogenase Activities of Submitochondrial Particles.* It was stated earlier that TPNH was capable of reducing electron carriers in preparations of complex I, but not in preparations of complex II, III, or IV. Furthermore, it was found that complex I preparations can catalyze a slow oxidation of TPNH by ferricyanide (Table II) (see Hatefi and Stempel, 1969, for DPNH–ferricyanide reductase activity of complex I).[4] Complexes II and III were completely devoid of TPNH–ferricyanide reductase activity, and the activity of complex IV preparations was extremely small (Table II). When the four complexes were tested for TPNH → DPN transhydrogenase activity, a somewhat similar pattern was observed (Table III). Complex I showed a TPNH → AP-DPN transhydrogenase activity of 0.3 μmol $min^{-1}$ $mg^{-1}$ of protein at 38°, complex II had no such activity, and the comparable activities of complexes III and IV were, respectively, 0.06 and 0.03. These results suggest, therefore, that both TPNH dehydrogenase and TPNH → DPN transhydrogenase activities of ETP fractionate more into complex I preparations than into those of the other three complexes. The association of TPNH dehydrogenase activity with complex I is also in agreement with the rotenone–piericidin sensitivity of TPNH oxidation by ETP.

It is important to note that the TPNH → DPN transhydrogenase activity of complex I is less than that of ETP, even though, on the basis of DPNH dehydrogenase–flavine, complex I represents a tenfold purification as compared to ETP. In part, the lower transhydrogenase activity of complex

[3] In the present studies no attempt was made to distinguish between iron–sulfur centers 3 and 4.

[4] Regarding the TPNH–ferricyanide reductase activity of complex I, it should be mentioned that Tottmar and Ragan (1971) have also observed that a (complex I)-like DPNH dehydrogenase isolated from *T. utilis* exhibited a TPNH–ferricyanide reductase activity, which was about 1% of the ferricyanide reductase activity with DPNH as substrate.

TABLE II: TPNH Dehydrogenase Activities of the Four Electron-Transfer Complexes.

| Enzyme | μmol of Ferricyanide Reduced/min$^{-1}$ × mg$^{-1}$ of Protein |
|---|---|
| Complex I | 1.7 |
| Complex II | 0.0 |
| Complex III | 0.0 |
| Complex IV | 0.2 |

I is due to the presence of bile salts, which inhibit the transhydrogenase reaction. However, the lower activity of complex I as compared to ETP is also because transhydrogenase activity is found in other mitochondrial fractions, which are intermediate in the isolation of the other three complexes. This observation suggests that TPNH → DPN transhydrogenase is not firmly bound to complex I and is removable by detergents. Kaplan (1967) has shown already that a transhydrogenase preparation can be isolated from mitochondria in the presence of digitonin.

It is seen in Table III that both ETP and complex I are also capable of catalyzing DPNH → AP-DPN transhydrogenation, and that the relative specific activities of complex I and ETP are in accord with their relative contents of DPNH dehydrogenase–flavine. Indeed, when complex I was resolved with 2.5 M urea (Hatefi and Stempel, 1967; Davis and Hatefi, 1969), it ws found that the only fraction containing DPNH → AP-DPN activity was the soluble DPNH dehydrogenase. However, neither the DPNH dehydrogenase nor the soluble iron–sulfur protein, isolated after urea-induced resolution of complex I, had any TPNH → DPN transhydrogenase activity.

Another point of interest with regard to the transhydrogenase activity of complex I is that it is inhibited by palmitoyl coenzyme A, which was shown by Ernster and coworkers (Rydström *et al.*, 1971b) to inhibit the transhydrogenase activity of submitochondrial particles. We have found that palmitoyl-CoA inhibits both DPNH and TPNH oxidation by ETP, but that the transhydrogenase is more affected. The left-hand trace in Figure 7 shows reduction of chromophores at 475 minus 510 nm in piericidin-treated ETP by TPNH, DPNH, and succinate. It is seen that each substrate leads to a certain degree of bleaching at 475 minus 510 nm.

TABLE III: Transhydrogenase Activities of ETP and Complex I.

| Transhydrogenation | Enzyme Source | Act. (μmol min$^{-1}$ × mg$^{-1}$ of Protein) |
|---|---|---|
| TPNH → AP-DPN | ETP | 0.8 |
| TPNH → AP-DPN | Complex I | 0.3 |
| TPNH → AP-DPN | Complex II | 0.00 |
| TPNH → AP-DPN | Complex III | 0.06 |
| TPNH → AP-DPN | Complex IV | 0.03 |
| DPNH → AP-DPN | ETP | 0.5 |
| DPNH → AP-DPN | Complex I | 3.9 |
| DPNH → AP-TPN | Complex I | 0.04 |
| TPNH → AP-TPN | Complex I | <0.03 |

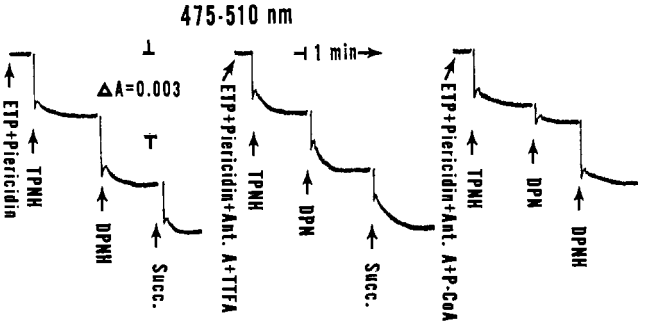


FIGURE 7: Effect of palmitoyl-CoA on reduction of chromophores at 475 minus 510 nm in ETP *via* TPNH → DPN transhydrogenation. Conditions: ETP, 2.2 mg of protein/ml; TPNH, 60 μM; DPNH, 60 μM; DPN, 140 μM; sodium succinate, 1.75 mM; piericidin A, 5.3 μM; antimycin A, 1 μM; 2-theonyltrifluoroacetone (TTFA), 1 mM; palmitoyl-CoA (P-CoA), 200 μM.

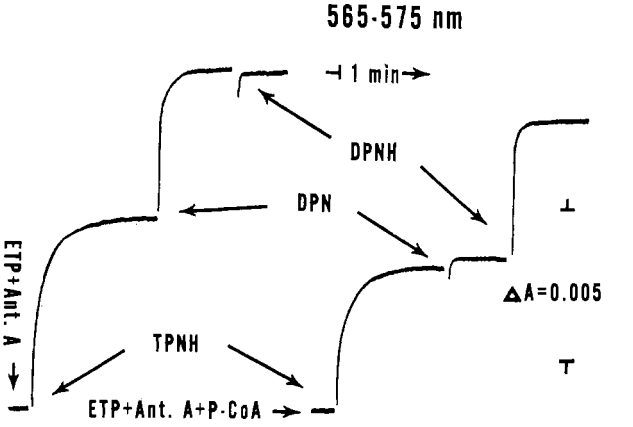


FIGURE 8: Effect of palmitoyl-CoA on cytochrome *b* reduction in ETP *via* TPNH → DPN transhydrogenation. Conditions: ETP, 0.78 mg/ml; antimycin A, palmitoyl-CoA, TPNH, DPNH, and DPN as in Figure 7.

In the middle trace DPNH is replaced by DPN, which *via* TPNH → DPN transhydrogenase becomes reduced and then causes additional bleaching at 475 minus 510 nm. For control, the effect of succinate is also shown. In addition to piericidin, the ETP used in this experiment was also treated with 2-thenoyltrifluoroacetone and antimycin A to show that these inhibitors had no effect on the extent of bleaching afforded at this wavelength pair by TPNH, DPNH, and succinate (see also data published in Hatefi, 1973a). However, when palmitoyl-CoA was present (right-hand trace), TPNH and DPNH reduction of chromophores at 475 minus 510 nm were not altered, but transhydrogenation, as shown by the DPN effect, was inhibited. Figure 8 shows similar results concerning the effect of palmitoyl-CoA on cytochrome *b* reduction in antimycin-treated ETP upon addition of TPNH followed by DPN. It is seen in the left-hand trace that under the particular conditions used (see Discussion) TPNH reduces roughly half of the total cytochrome *b*,[5] which is reducible by DPNH (or succinate, see Hatefi, 1973a) in antimycin-treated ETP. When DPN or DPNH is then added, the remainder of cytochrome *b* is reduced. The right-hand trace shows TPNH and DPNH reduction of *b* cytochromes, in palmitoyl-CoA-treated particles, but the reduction in-

[5] This species of cytochrome *b* has been shown to be $b_K$ (single α peak at 559.5 nm at 77°K; 563 nm at 295°K) (Hatefi, 1973a; Davis *et al.*, 1972).

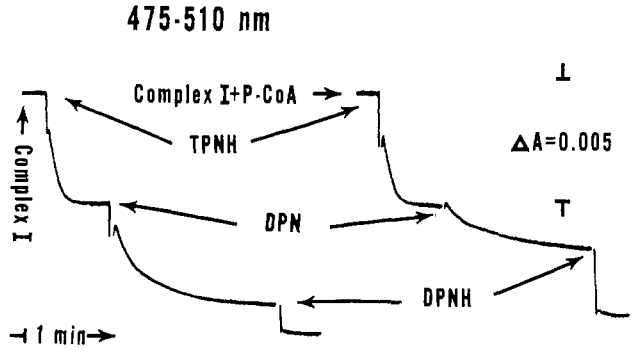


FIGURE 9: Effect of palmitoyl-CoA on reduction of chromophores at 475 minus 510 nm in complex I *via* TPNH → DPN transhydrogenation. Conditions: complex I, 0.94 mg of protein/ml in the presence of 0.1% (v/v) Triton X-100; TPNH, 60 μM; DPNH, 60 μM; DPN, 175 μM; palmitoyl-CoA, 280 μM.

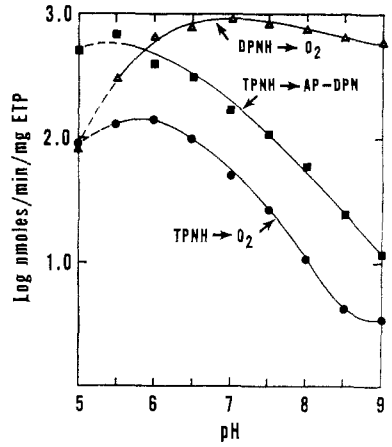


FIGURE 10: pH dependence of TPNH oxidase, TPNH → AP-DPN transhydrogenase and DPNH oxidase activities of ETP. Conditions: oxidase activities were measured in the presence of 2 mM DPNH or TPNH, 0.25 M sucrose, 100 mM sodium phosphate for pH values 6–9, and 100 mM sodium acetate for pH values 5.0 and 5.5. ETP concentration was 2.16 mg/ml for the TPNH oxidase, and 0.216 mg/ml for the DPNH oxidase assays. The transhydrogenase reaction was measured by the Aminco-Chance spectrophotometer at 400 minus 450 nm. The extinction coefficient used for AP-DPNH at 400 nm was calculated from the data of Siegel *et al.* (1959) to be approximately 2300 l. $mol^{-1}$ $cm^{-1}$. Media were the same as in the oxidase assays. Dotted lines indicate uncertainty about the pH 5 rates, because of possible acidity damage to ETP. The ordinate refers to nmoles of TPNH or DPNH oxidized $min^{-1} \times mg^{-1}$ of ETP protein at 30°.

duced by DPN, as a result of TPNH → DPN transhydrogenation, is inhibited. That TPNH → DPN transhydrogenase activity of complex I responds to palmitoyl-CoA in a manner similar to that shown with ETP (Figure 7) is demonstrated in Figure 9.

The possibility that TPNH dehydrogenation and transhydrogenation are catalyzed by the same mitochondrial enzyme is most persuasively suggested, however, by the similar responses of TPNH oxidase and TPNH → AP-DPN transhydrogenase activities of ETP to pH (Figure 10). The similarity between the pH-dependence curves of these two reactions is readily apparent from Figure 10. However, what is rather unusual and significant is that both the TPNH oxidase and the TPNH → AP-DPN transhydrogenase activities fall sharply as the pH of the medium is raised from pH 6 to 9. It might be noted that the ordinate scale in Figure 10 is logarithmic, and that activity differences between pH 6 and 9 for TPNH oxidase and TPNH → AP-DPN transhydrogenase reactions are, respectively, 42- and 35-fold. For comparison, the pH–response curve of the DPNH oxidase reaction with the same ETP preparation is also shown in Figure 10. It is seen that the pH optimum of this reaction is roughly one pH unit higher than those of the above reactions, and that the shape of the pH–activity curve of the DPNH oxidase reaction is completely different from those concerned with TPNH oxidation.

## Discussion

Since direct TPNH oxidation by the mitochondrial respiratory chain had not been demonstrated previously, and inner membrane preparations contain pyridine nucleotide transhydrogenase, the validity of our studies depend on excluding DPN intervention during TPNH oxidation. Three possible sources of DPN are (a) DPN contamination in the added TPNH, (b) particle-bound DPN in ETP, $ETP_H$, and complex I, and (c) enzymatic conversion of TPN to DPN by a phosphatase (Ernster *et al.*, 1969). However, there are several independent lines of evidence against DPN involvement in our studies. (1) As described in the section on Methods and Materials, the possible presence (or enzymatic production from TPN) of DPN in TPNH and in the assay systems involving large amounts of various particles was checked in the presence of alcohol and alcohol dehydrogenase or 3-hydroxybutyrate. The results showed a maximum amount of 0.2 nmol of DPN/mg of submitochondrial particles, and essentially none in complex I and TPNH. Direct experiments showed that 0.2 nmol of DPN/mg of ETP could account for only about 8% of the TPNH oxidase rates. In addition, these studies excluded rapid enzymatic conversion of TPN or TPNH to DPN. The absence of such an activity in beef heart submitochondrial particles has been reported previously (Ernster *et al.*, 1969). (2) In Figures 1B and 2A, the amounts of TPN reduced after addition of ATP were, respectively, 18 and 12 nmol. Addition of oxidized glutathione and glutathione reductase, after TPNH production was inhibited, caused complete reoxidation of TPNH precisely to the level before ATP addition. Had even one-tenth as much DPN been reduced in these experiments, it would not have been oxidized (especially in the experiment of Figure 2A, where rutamycin was the inhibitor) by glutathione and glutathione reductase, and would not have allowed the absorbance at 340–374 nm to return completely to the level before ATP addition. (3) As shown in Figures 3–7 and in Figure 9 (see, especially, the epr data of Figure 4B,C), fewer components of ETP and complex I were reduced by TPNH than by DPNH or TPNH plus DPN. These results, *per se*, exclude DPN involvement qualitatively. (4) In the experiments of Figures 7, 8, and 9, it is seen that palmitoyl-CoA inhibits transhydrogenation, but has little effect on the reductions afforded by TPNH or DPNH. Had the initial, TPNH-induced reduction of components in these experiments occurred by way of transhydrogenation to DPN, then palmitoyl-CoA would have inhibited this reaction, just as it did subsequently when DPN was added to the reaction mixtures.

We feel that these independent lines of evidence exclude DPN involvement, and permit of the following conclusion. The mitochondrial inner membranes can oxidize TPNH not only by transhydrogenation to DPN, but also *directly* through the electron transport system. This is not particularly surprising, because many pyridine nucleotide linked dehydro-

genase systems have such a dual catalytic capability. It has already been mentioned above that the mitochondrial DPNH dehydrogenase can also catalyze DPNH → AP-DPN transhydrogenation, and an interesting example of a TPNH-linked enzyme capable of both TPNH dehydrogenation and TPNH → AP-TPN transhydrogenation is that of dihydrofolate reductase. The transhydrogenase activity of this enzyme is dependent upon the presence of dihydro- or tetrahydrofolate, and is inhibited by the antifolate compound, amethopterin (Huennekens *et al.* (1970).

Perhaps a major reason that TPNH oxidation by submitochondrial particles had remained so long unnoticed is that at neutral pH (7.0–7.5) this activity is only 4–5% of the DPNH oxidase activity of submitochondrial particles (at pH 6.0 and 30°, the TPNH oxidase rate shown in Figure 10 was 145 nmol $min^{-1}$ $mg^{-1}$ of ETP protein, *i.e.*, 25% of the DPNH oxidase rate at this pH). It is doubtful that at neutral pH the low rates of TPNH oxidation and TPN reduction by the electron-transport system are significant for the physiology of mitochondria. Certainly, the transhydrogenase-catalyzed rates for both TPNH oxidation and TPN reduction are severalfold faster (Rydström *et al.*, 1971a; Hatefi, 1973a), and very likely predominate in maintaining mitochondrial TPN–TPNH balance. Another important consideration is that $K_m^{TPNH}$ in the oxidase system (*i.e.*, in the absence of DPN) is 550 μM,[6] whereas Teixeira da Cruz *et al.* (1971) have shown that in the TPNH → DPN transhydrogenase reaction $K_m^{TPNH}$ is only 20 μM. However, from the evolutionary standpoint, direct electron transfer between TPN–TPNH and the respiratory chain might be rather important as it is reminiscent of the photosynthetic electron-transport system. The other examples of similarity between the two systems are (a) the mitochondrial $b$–$c_1$ (Hatefi and Hanstein, 1972), the chromatophore $b$–$c_2$ (Baltscheffsky, 1969), and the chloroplast $b_6$–$f$ segments (Buchanan and Arnon, 1970), all of which appear to include sites for energy coupling and antimycin inhibition, and (b) the succinate dehydrogenases of bovine heart mitochondria (Davis and Hatefi, 1971) and *Rhodospirillum rubrum* chromatophores, whose similar catalytic and molecular properties and cross-reconstitution activities have been shown elsewhere (Hatefi *et al.*, 1972).

The finding that both the TPNH dehydrogenation site and the TPNH → DPN transhydrogenase are present in the complex I segment of the respiratory chain, and in proximity to DPNH dehydrogenase, is rather interesting. The pH-dependence data of Figure 10 strongly suggest that the same enzyme system might be responsible for both TPNH dehydrogenation and transhydrogenation, just as DPNH dehydrogenase is capable of both DPNH → AP-DPN transhydrogenation and electron transfer from DPNH to the respiratory chain.[7] If this possibility should prove to be correct, then the enigma of energy-linked reduction of TPN by DPNH will become amenable to more incisive experimental scrutiny, especially with regard to the mechanism by which energy transfer and utilization occur in conjunction with DPNH → TPN transhydrogenation. In addition, oxidative phosphorylation studies, similar to those carried out by Schatz and Racker (1966) for the DPNH to ubiquinone span, might yield information regarding the number and the locations of coupling sites between TPNH and the ubiquinone–cytochrome $b$ region of the electron-transport system.

Other results of considerable interest which have emerged from the study of TPNH oxidation by the respiratory chain are as follows. (a) It is known that in nonphosphorylating systems succinate and DPNH reduce only cytochrome $b_K$. However, they reduce both $b_K$ and $b_T$ when the particles are treated with antimycin A (Davis *et al.*, 1972). Studies with TPNH as substrate have shown, however, that the latter is not always the case. In the presence of low levels of TPNH (*e.g.*, $<0.5K_m$) and at pH $\geq 7.5$, only $b_K$ reduction can be demonstrated in antimycin-treated ETP, but $b_T$ can be reduced also in such a system upon further addition of TPNH, DPNH, succinate, or DPN (the latter produces DPNH from excess TPNH *via* transhydrogenase) (Hatefi, 1973a). The reason that in antimycin-treated ETP under the above conditions cytochrome $b_T$ reduction cannot be demonstrated appears to be related to (a) the slow rate of electron transfer from TPNH to the respiratory chain, and (b) the slow rate of $b_K$ reoxidation, as compared to $b_T$ reoxidation, through the antimycin leak. Similar results were obtained when the rate of substrate oxidation was controlled by adding either low levels of succinate or DPNH in the presence of 2 mM Seconal, which caused partial inhibition of electron transfer from DPNH to the ubiquinone–cytochrome $b$ segment of the respiratory chain. These findings are of interest with regard to electron-transfer pathways and control mechanisms at the Q–b region. Further, Chance *et al.* (1970) have suggested that the apparent redox potential change of $b_T$ (from −35 to +245 mV: Wilson and Dutton, 1970) upon treatment of mitochondria with ATP might represent energy conservation at coupling site II. Therefore, a slow oxidase reaction with TPNH, or other substrates as described above, might provide an appropriate system in which to see whether substantial $b_T$ reduction is necessary for site II coupling. (b) As mentioned above, resolution of complex I with urea or more potent chaotropes results in solubilization of a 70,000 mol wt iron–sulfur flavoprotein with DPNH dehydrogenase activity (Hatefi and Stempel, 1969; Davis and Hatefi, 1969). We have assumed that this preparation represents the DPNH dehydrogenase of the electron-transport system, despite the fact that, as compared to the membrane-bound enzyme, the soluble dehydrogenase shows a tenfold increase in $K_m$ for DPNH and a wide acceptor specificity with regard to quinoid and ferric compounds. That many membrane-bound enzymes undergo changes in catalytic properties upon isolation is well known (Hatefi, 1968), and Racker (1967) has suggested the term allotopic change for this all too common phenomenon. Singer and coworkers (Singer, 1966; Singer and Gutman, 1971) argue, however, that the above dehydrogenase is a "peptide fragment" of a large molecular weight ($6–8 \times 10^5$) preparation, which they have isolated and consider to be the true mitochondrial DPNH dehydrogenase. This preparation is quite comparable in flavine, iron, and labile sulfide content (hence in minimum molecular weight) to complex I. Both complex I and the Singer dehydrogenase have comparable ferricyanide reductase activities, but the latter is incapable of ubiquinone reduction, possibly because of lipid deficiency owing to its isolation in the presence of phospholipase A.

The results presented in this communication show clearly that TPNH reduces all the known components of complex I, except iron–sulfur center 1 and, apparently, flavine. In other words, the latter are the only respiratory chain components which are specifically reduced by DPNH. That iron–sulfur

[6] This value has been shown to be the same at the pH range 6.0 to 7.5. The earlier printed value of 55 μM (Hatefi, 1973a) was erroneous. It should have been 550 μM.

[7] Another possibility that should be considered is that electron transfer might also occur from DPNH to the respiratory chain by way of the TPNH dehydrogenase–transhydrogenase system.

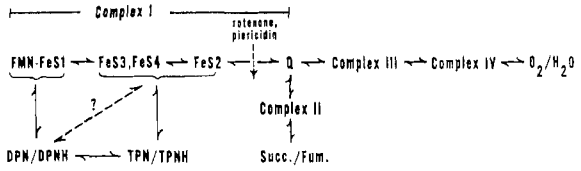


FIGURE 11: Scheme showing sites at which DPN–DPNH and TPN–TPNH appear to interact with the complex I segment of the electron-transport system. FeS1 to 4: respectively, iron–sulfur centers 1, 2, 3, and 4.

center 1 is most likely the iron–sulfur component of the small molecular weight DPNH dehydrogenase is indicated by its having the most negative redox potential of the four centers (Ohnishi *et al.*, 1972) and being the first center to accept electrons from DPNH (Orme-Johnson *et al.*, 1971). Thus, it may be concluded that complex I is actually a DPNH– and TPNH–ubiquinone reductase, of which the segment isolatable[8] as a 70,000 molecular weight, soluble protein, containing 1 mol of FMN, 4 g-atoms of Fe, and 4 mol of acid-labile sulfide/mol of protein, is specifically concerned with DPNH dehydrogenation. The electron transport scheme shown in Figure 11 summarizes the data presented in this paper with regard to the sites at which DPN–DPNH and TPN–TPNH appear to interact with the complex I segment of the respiratory chain.

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[8] Under conditions (neutral pH, moderate temperatures, no addition of proteolytic enzymes) which would not be expected to lead to peptide-bond hydrolysis.