Oxidation of NADPH by Submitochondrial Particles from Beef Heart in Complete Absence of Transhydrogenase Activity from NADPH to NAD*

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Treatment of submitochondrial particles (ETP) with trypsin at 0° destroyed NADPH → NAD (or 3-acetylpyridine adenine dinucleotide, AcPyAD) transhydrogenase activity. NADH oxidase activity was unaffected; NADPH oxidase and NADH → AcPyAD transhydrogenase activities were diminished by less than 10%. When ETP was incubated with trypsin at 30°, NADPH → NAD transhydrogenase activity was rapidly lost, NADPH oxidase activity was slowly destroyed, but NADH oxidase activity remained intact. The reduction pattern by NADPH, NADPH + NAD, and NADH of chromophores absorbing at 475 minus 510 nm (flavin and iron-sulfur centers) in complex I (NADH-ubiquinone reductase) or ETP treated with trypsin at 0° also indicated specific destruction of transhydrogenase activity.

The sensitivity of the NADPH → NAD transhydrogenase reaction to trypsin suggested the involvement of susceptible arginyl residues in the enzyme. Arginyl residues are considered to be positively charged binding sites for anionic substrates and ligands in many enzymes. Treatment of ETP with the specific arginine-binding reagent, butanedione, inhibited transhydrogenation from NADPH + NAD (or AcPyAD). It had no effect on NADH oxidation, and inhibited NADPH oxidation and NADH → AcPyAD transhydrogenation by only 10 to 15% even after 30 to 60 min incubation of ETP with butanedione. The inhibition of NADPH → NAD transhydrogenation was diminished considerably when butanedione was added to ETP in the presence of NAD or NADP. When both NAD and NADP were present, the butanedione effect was completely abolished, thus suggesting the possible presence of arginyl residues at the nucleotide binding site of the NADPH → NAD transhydrogenase enzyme. Under conditions that transhydrogenation from NADPH to NAD was completely inhibited by trypsin or butanedione, NADPH oxidation rate was ≥220 nmol min⁻¹ mg⁻¹ ETP protein at pH 6.0 and 30°. The above results establish that in the respiratory chain of beef-heart mitochondria NADH oxidation, NADPH oxidation, and NADPH → NAD transhydrogenation are independent reactions.

Recent studies in this laboratory (1-3) have shown that the electron transport system of bovine-heart mitochondria is capable of catalyzing the oxidation of NADPH in the absence of detectable amounts of NAD. This was contrary to the general belief that mitochondria do not possess a direct route for NADPH oxidation, and that this nucleotide is oxidized only by transhydrogenation to NAD, as catalyzed by the membrane-bound transhydrogenase enzyme, and subsequent oxidation of the NADH so formed by the normal NADH oxidase pathway of the respiratory chain (4, 5).

Our studies showed that the site of NADPH oxidation resides in complex I, but is different from that of NADH oxidation. Whereas NADH reduced the flavin and iron-sulfur centers 1, 2, 3, and 4 of complex I (1-3, 6, 7). NADPH was shown to reduce only center 2 and partially the centers 3 + 4 whose EPR signals overlap. Subsequent studies 1 indicated that this partial reduction was mainly (or possibly entirely) due to center 3. Thus, it appeared that at neutral pH NADPH reduces the components of complex I which exhibit oxidation-reduction potentials near zero (i.e. centers 2 and 3), but not those with low potentials (i.e. flavin, iron-sulfur center 1, and apparently center 4) (3, 6, 7). In agreement with these results, NADPH oxidation by the respiratory chain was inhibited by rotenone, piericidin A, antimycin A, and cyanide, but not by 2-thienyltrifluoroacetone at concentrations which this compound inhibits succinate oxidation. In addition, it was shown that phosphorylating submitochondrial particles catalyzed ATP-induced reverse electron transfer from succinate to NADP. This reaction was inhibited by uncouplers and by

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rotenone or piericidin A. The NADPH dehydrogenase and the NADPH → NAD transhydrogenase enzymes exhibited several common features: (a) both enzymes fractioned into complex I, (b) both were stereospecific for abstraction of the 4-B hydrogen of NADPH (3), (c) both showed a similar and unusually high response to pH (about 35% to 40-fold increase in catalytic activity as the pH of the assay medium was lowered from 9 to 6), and (d) both activities were inhibited by Mg²⁺.

In spite of the fact that NADPH did not reduce the flavin and iron-sulfur center 1 of the NADH oxidase pathway, the similar features of NADPH dehydrogenase and transhydrogenase made it desirable to demonstrate NADPH oxidation under conditions that the transhydrogenase reaction was clearly inoperative. Such evidence was actually presented in our earlier work (2, 3) with the use of palmitoyl coenzyme A, which inhibits transhydrogenation much more than NADPH oxidation. It was shown that palmitoyl-CoA-treated particles were able to accept electrons from NADPH or NADH for the reduction of respiratory chain components reduced by these nucleotides, but not from NADPH + NAD as a source of NADH (cf. Fig. 7 and 8 of Ref. 2). These results indicated that both NADH and NADPH oxidation could take place under conditions that transhydrogenation from NADPH to NAD was inhibited by palmitoyl CoA. However, these experiments were not designed to demonstrate the oxidation of substrate quantities of NADPH in a transhydrogenase-inhibited system, and were somewhat complicated by the fact that palmitoyl CoA at the concentrations needed for complete inhibition of transhydrogenation had detergent-like effects and resulted in considerable inhibition of NADPH and partial inhibition of NADH oxidation as well.

The present paper provides unambiguous evidence for the oxidation of NADPH by submitochondrial particles under conditions that transhydrogenation from NADPH to NAD is completely destroyed. This selective destruction is achieved by treating the particles with trypsin. Other results will show that the trypsin effect appears to be related to the presence of arginyl residues in the transhydrogenase enzyme, probably located at the enzyme active site. Thus, addition to the particles of the specific arginine binding reagent, butanedione, also resulted in the selective inhibition of the transhydrogenase reaction with little or no effect on the NADH or the NADPH oxidase activities.

METHODS AND MATERIALS

ETP (8) and complex I (9) were prepared according to the references given. The transhydrogenase assay for the reduction of AcPyAD by NADH or NADPH was carried out essentially according to Kaplan (10) as described elsewhere (2).

Assays for the oxidation of NADH, NADPH, or NADPH + NAD, the rate of which is controlled by the transhydrogenase reaction, were carried out polarographically at 30°, using a Clark electrode for measuring oxygen consumption. The assay medium consisted of 1.75 ml final volume of 0.25 m sucrose and 100 mM sodium phosphate or Tris sulfate, pH 7.0, was mixed with 0.1 mg of trypsin (grade B) from Calbiochem and butanedione from Aldrich Chemical Co. All other chemicals were the same as before (2) except for Mg²⁺.

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Butanedione treatment of ETP was carried out as follows. ETP at 35 to 60 mg of protein/ml of 0.25 mM sucrose and 50 mM sodium borate, pH 9.0, was treated with a given amount of butanedione and incubated at 20°. Control particles were treated similarly, except that instead of butanedione they received an equivalent volume of water. Samples were removed at intervals and assayed at 30° for the oxidation of NADH, NADPH, and NADPH + NAD, or for transhydrogenation from NADH or NADPH to AcPyAD as indicated above. In the polarographic experiments with butanedione-treated ETP, the concentrations of NADH, NADPH, and NAD in the reaction mixtures were, respectively, 0.75, 1.7, and 0.3 mM. ETP (50 µl) was added at a concentration of 1.14 to 1.71 mg/ml of the reaction mixture in order to obtain the same final pH in the reaction mixture in all the experiments. This pH was 6.55. The fast rate of NADH oxidation of such high concentrations of ETP was suitably adjusted by the chart speed of the recorder for convenient rate measurements. Experiments in which NAD and NADPH were added to ETP to protect against butanedione inhibition were conducted similarly, except that in the preincubation mixture the nucleotides were first added to the ETP suspension, followed immediately by butanedione.

The experiments of Figs. 3 and 4 were carried out as 22° essentially as before (2) using the Aminco-Chance dual wavelength spectrophotometer. Protein was estimated by the biuret method (11) in the presence of 1 mg of potassium decyloxyolate/ml.

Nicotinamide and 3-acetylpyridine nucleotides were obtained from P-L Biochemicals, trypsin (grade B) from Calbiochem, and butanedione from Aldrich Chemical Co. All other chemicals were the same as before (2) or reagent grade.

RESULTS

Effect of Trypsin—It has been shown by Juntti et al. (12) that the nicotinamide adenine dinucleotide transhydrogenase activity of submitochondrial particles is extremely sensitive to treatment of the particles with trypsin. Taking advantage of the exceptional sensitivity of the transhydrogenase reaction to trypsin, we were able to show that this reaction can be destroyed under conditions that the NADH oxidase and the NADPH oxidase activities of submitochondrial particles are essentially unaffected. As seen in Fig. 1 (left panel), when submitochondrial particles were treated at 0° with 0.1 mg of trypsin/mg of particle protein, the transhydrogenase activity as measured by the oxidation of NADPH + NAD (minus the
contribution of the oxidation rate of NADPH alone) was completely destroyed after 10 min, whereas NADH oxidase activity was unaffected and the NADPH oxidase activity diminished initially by only 8% and thereafter remained unchanged even up to 120 min. The activity assays were performed at pH 6.0, which is close to optimal for the transhydrogenase and the NADPH oxidase reactions. At this pH, the oxidation rates of NADH, NADPH, and NADPH + NAD at zero time were, respectively, 1140, 240, and 530 nmol min⁻¹ mg⁻¹ of ETP protein. The trypsin effect was the same when the activity assays were conducted at pH 7.0 or 7.5. At pH 7.0 the initial oxidation rates of NADH, NADPH, and NADPH + NAD were, respectively, 1580, 74, and 320 nmol min⁻¹ mg⁻¹ of ETP protein. The right-hand part of Fig. 1 shows the effect of trypsin on the above activities when the particles were incubated at 30°C with 0.1 mg of trypsin/mg of transhydrogenase and the NADPH oxidase reactions. At this pH, NADH oxidase remained unaffected, but now NADPH oxidase also became slowly inhibited. After 55 min, the latter activity was destroyed by 64%. At higher trypsin concentrations (i.e., ≥0.2 mg of trypsin/mg of ETP protein) and at temperatures of ≥20°C, NADH oxidase activity also began to decline after about 1 hour of incubation. In these and the experiments described hereafter, particles untreated with trypsin or other inhibitors, but otherwise similarly treated and incubated in parallel, showed no loss of activity in any of the assays.

The sensitivity of the transhydrogenase reaction to trypsin treatment of the particles was tested also by the spectrophotometric procedure of Kaplan (10), in which the reduction of AcPyAD by NADH and NADPH was measured at 375 nm in the presence of submitochondrial particles terminally blocked with KCN. In these experiments, it should be noted that the NADPH → AcPyAD transhydrogenation is catalyzed by the nicotinamide adenine dinucleotide transhydrogenase enzyme, whereas NADH → AcPyAD transhydrogenation is catalyzed by the mitochondrial NADH dehydrogenase and is shown here for comparison. As seen in Fig. 2, the results were in agreement with the polarographic data of Fig. 1. Thus, in the direct transhydrogenase assay, the ability of trypsin-treated particles to catalyze the reduction of AcPyAD by NADPH was completely ablated between 8 and 15 min of incubation at 0°C, whereas by comparison NADH → AcPyAD transhydrogenation was diminished by only 10%. The results of Figs. 1 and 2 show clearly (a) that NADH oxidase, NADPH oxidase, and NADPH to NAD transhydrogenase activities of submitochondrial particles can be distinguished from one another by their different degrees of sensitivity to trypsin, (b) that submitochondrial particles can oxidize NADPH at appreciable rates (e.g., 220 nmol min⁻¹ mg⁻¹ of ETP protein at pH 6.0) under conditions that the transhydrogenase reaction from NADPH to NAD is completely destroyed, and (c) that trypsin specifically destroys transhydrogenase from NADPH to NAD (or AcPyAD) and has little effect on transhydrogenation from NADH to NAD (or AcPyAD), which is catalyzed by the mitochondrial NADH dehydrogenase enzyme.

It has been shown in previous studies (1-3) that at the wavelength pair 475 minus 510 nm, the combined reduction of flavin and iron-sulfur proteins can be monitored in mitochondria and derivative particles when the reduction of cytochromes is blocked by an appropriate inhibitor. Thus, when NADH is added to rotenone-treated particles, the bleaching at 475 minus 510 nm is a measure of the reduction of FMN and iron-sulfur centers 1, 2, 3, and 4 which are located on the substrate side of the rotenone block. Since FMN, iron-sulfur center 1, and apparently iron-sulfur center 4, are not detectably reduced by NADPH, addition of this substrate to rotenone-treated submitochondrial particles or to complex I was shown to result in 50 to 65% bleaching at 475-510 nm as compared to the bleaching afforded by NADH. Addition of NAD to the system partially reduced by NADPH resulted in further bleaching. This was apparently the procedure of transhydrogenase action, yielding NADH from excess NADPH + NAD, and subsequent bleaching by NADH of FMN and iron-sulfur centers 1 and 4. It was also shown that the NAD-induced, but not the NADPH-induced, bleaching could be inhibited by appropriate levels of palmitoyl coenzyme A, which appeared to have a greater effect on the NADPH → NAD transhydrogenase than on the NADPH dehydrogenase activity of submitochondrial particles or complex I.

Figs. 3 and 4 show the effect of trypsin on the bleaching afforded at 475-510 nm in submitochondrial particles (Fig. 3) and complex I (Fig. 4) by addition of NADPH, NAD (in effect NADH produced from NADPH + NAD as a result of transhydrogenase action), and NADH. As seen in Fig. 3, left trace, addition of NADPH to submitochondrial particles not treated with trypsin resulted in a certain amount of bleaching at 475–510 nm, which was increased upon further addition of NADH. In the middle trace of Fig. 3 NADPH addition was followed by NAD instead of NADH. It is seen that the same amount of additional bleaching took place, but at a slower rate, which is indicative of NADH production from NADPH + NAD by the transhydrogenase reaction. Further addition of NADH caused no additional bleaching. The result of trypsin treatment of the particles after 17 hours at 0°C is shown in the right-hand trace of Fig. 3. It is seen that the bleaching afforded by addition of NAD is inhibited, but the effects of NADPH and NADH are essentially unchanged. In agreement with the data described above, these results indicate that trypsin treatment preferentially destroyed the transhydrogenase reaction. Consequently, introduction of NAD into a system containing NADPH did not result in NADH production and additional bleaching at 475–510 nm. That the chromophores responsible for the additional bleaching (apparently FMN and iron-sulfur centers 1 and 4) are not destroyed by such prolonged trypsin

*The higher degree of bleaching is observed at pH values below neutrality (e.g., pH 6 to 6.5).
105% and 82%, respectively, of the corresponding activities of control particles at zero time. Assays were performed in the same buffer mixture at pH 7.5 and 22° in the presence of 7 μm rotenone. The slow and incomplete reduction afforded by NAD was essentially the same after 14 min (Fig. 4, third trace from left) and after 60 min (not shown), but became perceptibly diminished after 19.5 hours at 0°. This prolonged treatment of complex I with trypsin also resulted in about 10% diminution of the extent of reduction afforded by NADPH, which could be due to destruction of a chromophore. However, the rates of reduction by NADPH and NADH, if also affected, were not of such a magnitude to be detectable in these assays. The control preparation of complex I untreated with trypsin, but kept at 0° for 19.5 hours, showed 9% increase in the extent of bleaching afforded by NADPH and 10% increase in the total bleaching afforded after additions of NADPH, NAD, and NADH. Otherwise, it appeared the same as the zero time control. The reason for this slight increase in the degree of bleaching appeared to be due to better dispersion of complex I particles upon standing in solution at 0°. The results of Figs. 3 and 4 are in full agreement with our previous work (2, 3) and the data presented in Figs. 1 and 2 regarding the effect of trypsin on the NADPH → NAD transhydrogenase reaction. Since the bleaching at 475–510 nm observed in Fig. 3 and 4 depends on very few turnovers of the enzymes concerned, these data also show how effectively trypsin can destroy NADPH → NAD transhydrogenation in ETP and complex I.

Effect of Butanedione—It has been shown by Riordan (10) that treatment of carboxypeptidase A with butanedione in borate buffer markedly decreased peptidase activity. This activity decrease was correlated with the modification of a single arginyl residue. Apparently, butanedione reacts reversibly with the guanido group of arginine to form a 4,5-dimethyl-4,5-dihydroxy-2-imidazoline derivative. Borate then reacts with this cis-diol to form a more stable complex. Lange and his colleagues (14) have shown that alcohol dehydrogenases from human liver, horse liver, and yeast can also be inhibited by treatment with butanedione in the presence of borate buffer. These studies indicated the involvement of arginyl residues as components of the NADH binding sites in these alcohol dehydrogenases. The authors feel that arginyl residues are probably the positively charged recognition sites of a large number of enzymes for anionic substrates and other ligands. In the case of alcohol dehydrogenases, they suggest that the enzyme arginyl residues interact with the pyrophosphate moiety of NADH.

In view of the above findings, the trypsin sensitivity of the transhydrogenase reaction suggested that this effect might be related to the presence of trypsin-susceptible arginyl residues in the enzyme. Thus, the effect of butanedione in borate buffer was tested on the activities studied in Figs. 1 and 2. Fig. 5 shows the effect of butanedione on the NADH oxidase, the NADPH oxidase and the NADPH + NAD oxidase activities of ETP. The particles suspended in 0.25 M sucrose and 50 mM borate buffer, pH 9.0, were incubated at 25° with 10 mM butanedione. Samples were removed at the times indicated and assayed polarographically as described under “Methods and Materials.” It is seen in Fig. 5 that NADH oxidase activity was unaffected by 10 mM butanedione and NADPH oxidase was inhibited only slightly (13% after 33 min incubation), but that transhydrogenase activity was completely abolished in about 20 to 25 min. Spectrophotometric results of transhydrogenation from NADH and NADPH to AcPyAD were also similar to those obtained in Fig. 2 with trypsin-treated particles. Thus as shown in Fig. 6, treatment of ETP in borate buffer with 10 mM butanedione resulted in a nearly complete

![Fig. 3. Effect of trypsin treatment of ETP at 0° on the reduction of chromophores absorbing at 475 minus 510 nm by NADPH, NADPH + NAD, and NADH. ETP at 29 mg of protein/ml of 0.25 M sucrose and 100 mM sodium phosphate, pH 7.0, was treated with 0.1 mg of trypsin/mg of ETP protein. Assays were performed in the same buffer mixture at pH 7.5 and 22° in the presence of 7 μm rotenone.](http://www.jbc.org/)

![Fig. 4. Effect of trypsin treatment of complex I at 0° on the reduction of chromophores absorbing at 475 minus 510 nm by NADPH, NADPH + NAD, and NADH. Complex I at 31.6 mg of protein/ml was treated with trypsin as in Fig. 3. The experiments were performed at 22° in 100 mM Tris sulfate, pH 7.0, containing 0.1% Triton X-100.](http://www.jbc.org/)
were respectively 940 and 966 nmol min\(^{-1}\) mg\(^{-1}\) of ETP protein. Assays were performed in 0.25 M Tris sulfate at a final pH of 6.55. Zero time oxidation rates for NADH and NADPH + NAD were the same as in Fig. 7. For NADH, the oxidation rates at zero time and 50 min were 940 and 960 nmol min\(^{-1}\) mg\(^{-1}\) of ETP protein.

Fig. 5. Effect of preincubation of ETP with 10 mM butanedione in the presence of borate buffer on the oxidation rates of NADH, NADPH, and NADPH + NAD. Incubation of ETP with butanedione was carried out at 25°C as described under “Methods and Materials.” Assays were performed in 0.25 M Tris sulfate at a final pH of 6.55. Zero time oxidation rates for NADPH and NADPH + NAD were the same as in Fig. 7. For NADH, the oxidation rates at zero time and 50 min were, respectively 940 and 960 nmol min\(^{-1}\) mg\(^{-1}\) of ETP protein.

Fig. 6. Effect of preincubation of ETP with 10 mM butanedione in the presence of borate buffer on transhydrogenase activities from NADH and NADPH to AcPyAD. Assays were performed at 30°C and pH 6.3. At zero time, the transhydrogenase activities in the absence of butanedione were 700 and 775 nmol min\(^{-1}\) mg\(^{-1}\) of ETP protein for NADPH → AcPyAD and NADH → AcPyAD, respectively.

loss of NADPH → AcPyAD transhydrogenase activity after 50 to 60 min, but in only about 10% loss of NADH → AcPyAD transhydrogenase activity. Both activities remained unchanged in the absence of butanedione. The combined results of transhydrogenase inactivation by trypsin and by butanedione are consistent, therefore, with the possible presence of 1 or more arginyl residues in the enzyme.

If as in the case of the alcohol dehydrogenases studied by Lange and co-workers (14) the arginyl residue(s) of the transhydrogenase enzyme is located at the nucleotide binding site, then it might be expected that the presence of the nucleotide substrates in the butanedione incubation mixture would prevent butanedione binding and inhibition. The results of such an experiment are shown in Fig. 7. It is seen that preincubation of ETP in borate buffer with 5 mM butanedione leads after 50 min to nearly complete inhibition of NADPH → NAD transhydrogenase activity with only about 10% loss of NADPH oxidase activity during the same period. Addition of 50 mM NAD or NADP to the preincubation mixture immediately before the addition of butanedione resulted in considerable protection. Addition of 100 mM NADP (or NAD, not shown in Fig. 7) gave even greater protection, while the addition of 50 mM NAD plus 50 mM NADP almost completely prevented the butanedione effect. These results suggest (a) that butanedione interacts with groups (very likely arginyl residues) at the nucleotide-binding site of the transhydrogenase enzyme, and (b) that consistent with the function of the enzyme the combined addition of NAD and NADP offers better protection (possibly because of cooperative binding affinity increase) than either nucleotide at the same total concentration. First order plots of the results of Figs. 5, 6, and 7 indicate a linear (first order) decay of transhydrogenase activity up to about 20 min incubation. Thereafter (i.e. in Figs. 6 and 7), a second first order decay ensues with a rate constant which is 36% of the total rate constant of the first 20-min decay. These results might mean that the transhydrogenase enzyme contains more than 1 arginyl residue at its active site, and that butanedione interaction with the first arginyl residue is faster and more inhibitory (about 70% of the total activity appears to be inhibited by the faster route).

Effect of Palmitoyl Coenzyme A—It has been shown by Rydström et al. (15) that palmitoyl coenzyme A inhibits the mitochondrial transhydrogenase reaction. Our previous studies have confirmed this, and demonstrated that at high concentrations palmitoyl-CoA also inhibits the NADPH and NADH oxidase activities of submitochondrial particles. These studies further showed that under appropriate conditions the transhydrogenase activity of submitochondrial particles or complex I could be completely inhibited while electron transfer from NADPH or NADH to the appropriate electron carriers of these systems still took place (2).

Fig. 8 shows the differential effect of 22 μM palmitoyl-CoA on NADH oxidase, NADPH oxidase, and NADPH + NAD oxidase activities of submitochondrial particles as a function of pH. It is seen that NADH oxidation is essentially unaffected by 22 μM palmitoyl-CoA over the pH range 7 to 8.5. NADPH oxidation rate is 35 to 40% inhibited, but the effect of pH change from 7 to 8.5 on palmitoyl-CoA inhibition of NADPH oxidation is insignificant. The transhydrogenase reaction as measured by the oxidation of NADPH + NAD (minus the contribution of NADPH oxidation by itself) showed a completely different picture, however. At pH 7.0, the reaction was 54% inhibited by 22 μM palmitoyl-CoA. This inhibition increased as the pH of the reaction mixture was raised, and reached to 83% inhibition at pH 8.5. In agreement with the results discussed above on the effects of trypsin and butanedione, the inhibitory effect of palmitoyl-CoA as a function of pH also demonstrates that the characteristics of the NADPH oxidase reaction of submitochondrial particles fit neither the NADH oxidase nor the NADPH → NAD transhydrogenase reactions.

DISCUSSION

It has been shown in the preceding section that treatment of submitochondrial particles with trypsin or with butanedione in
centers 1, 2, 3, and 4 of complex I, while NADPH appears to increase 35- to 40-fold as the pH of the assay medium is increased 3 to 4-fold (maximum at pH 7.0); reductase activity varied only by 2-fold (maximum at pH 7.0).

These similarities suggested that NADPH dehydrogenation in the absence of NAD might be a property of the transhydrogenase enzyme. NADPH oxidation and NADPH → NAD transhydrogenase activities of ETP. Assays were performed in 0.25 M sucrose containing 50 mM Tris acetate and another 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer at the pH values given. Each point is an average of several measurements in each buffer. Absolute activities for NADH oxidation were slightly higher in Hepes than in Tris acetate buffer; the reverse was true for NADPH and NADPH → NAD oxidation rates.

On the other hand, we had shown previously that the mitochondrial NADPH dehydrogenase is a flavoprotein, just as nicotinamide adenine dinucleotide dehydrogenases capable of interacting with single electron acceptors generally are. In this respect, it might be added that (a) treatment of complex I with NADPH does not produce a flavin free radical signal, (b) all the flavin of complex I is FMN, and more than 80% of this flavin can be isolated from complex I as a component of the soluble NADPH dehydrogenase, which exhibits no NADPH dehydrogenase activity (2, 16, 17), and (c) the difference spectrum of NADPH-treated minus NADH-treated complex I showed a flavin spectrum amounting to 1.2 nmol/mg of protein (2). This was about 90% of the flavin of the complex I preparation used in this experiment. These results are not evidence against the mitochondrial NADPH dehydrogenase being a flavoprotein. However, they do show that in complex I at least 90% of the flavin is specifically reduced by NADH, and that if any NADPH dehydrogenase flavin is present its molar concentration is not more than one-tenth that of the other recognized electron carriers of complex I. On the other hand, if the mitochondrial NADPH dehydrogenase should prove not to be a flavoprotein, then it might be a rather unique enzyme. This is because its substrate is a hydride donor and, so far as is known, its acceptor is an iron-sulfur center, which is a single electron acceptor, and its prosthetic group is a thiol or disulfide, neither of which is known as a biological device for “stepping-down” a 2-electron donor to a 1-electron acceptor. Sulphydryl proteins with diaphorase-like activity are known, e.g. glyceroldehyde-3-phosphate dehydrogenase and methemoglobin reductase (18-20). The former can catalyze the oxidation of NADH by DCIP, and the latter can reduce DCIP with either NADH or NADPH as substrate. However, so far as known neither enzyme reacts with a single electron acceptor. Furthermore, there is no evidence that DCIP reduction involves semiquinone intermediates. Benitez and Allison (19) have investigated the mechanism of DCIP reduction by glyceroldehyde-3-phosphate dehydrogenase. Their proposed scheme involves hydride ion transfer from NADH to DCIP, both enzyme-bound, followed by oxidation of the reduced DCIP by a second DCIP molecule.

The physiological significance of the NADPH oxidase pathway is difficult to rationalize, since NADPH is more rapidly oxidized and NADP is more rapidly reduced via the transhydrogenase enzyme in analogy with a number of enzymes which exhibit both nicotinamide adenine dinucleotide dehydrogenase and transhydrogenase activities. However, the data presented in this paper show that NADPH oxidation is essentially unaffected under conditions that NADPH → NAD transhydrogenation is completely destroyed. Thus, one obvious possibility is that the similarities between these two enzymatic activities are coincidental. Another possibility which cannot be ruled out by the available data is that NADPH dehydrogenation is a property of the transhydrogenase enzyme, and that trypsin and butanedione affect the transhydrogenase activity by modifying preferentially the NAD binding site. This possibility is not fully in accord, however, with the observation that NADP can protect against butanedione inhibition as well as NAD, but butanedione binding does not inhibit NADPH oxidation. The answer will probably have to wait until the elusive transhydrogenase enzyme is purified.

Even though it is known as methemoglobin reductase, this enzyme per se does not reduce methemoglobin with either NADH or NADPH as substrate.
drenase reaction. For that matter, why should the mitocho-
drial NADH dehydrogenase, as well as many other nicotina-
mide adenine dinucleotide dehydrogenases (to wit, dihydrofo-
late reductase as an example of a NADPH-linked enzyme, see
Ref. 21), have transhydrogenase activity? With the mitocho-
drial NADH dehydrogenase and the NADPH-linked dihy-
drofate reductase, transhydrogenation to AcPyAD and 3-
acetylpyridine adenine dinucleotide phosphate, respectively,
appear to be secondary reactions. Should transhydrogenation
and NADPH dehydrogenation turn out to be two functional
aspects of the mitochondrial NADPH → NAD transhydrogen-
ase enzyme, it is possible by reverse analogy that in this case
NADPH dehydrogenation is a secondary reaction. Regardless
of its physiological significance, direct oxidation of NADPH
and reduction of NADP (the latter is energy-linked) by the
respiratory chain are highly interesting processes. This is not
only in view of the mechanistic problems discussed above, but
also because studies to be described elsewhere have shown that
oxidative phosphorylation with either NADH or NADPH as
substrate yields phosphorus to oxygen ratios under optimal
conditions of 2.4 to 2.8. This suggests that both oxidation
pathways are linked to coupling site 1. However, since NADPH
appears to reduce only iron-sulfur centers 2 and 3 and bypass
FMN and iron-sulfur center 1, electron transfer from NADPH
to ubiquinone would have to involve a coupling site. This is
probably the best defined and least complicated segment of
the respiratory chain for the study of the mechanism of energy
conservation and coupling.

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Tokyo
Oxidation of NADPH by submitochondrial particles from beef heart in complete absence of transhydrogenase activity from NADPH to NAD.

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