ON THE REDUCTION OF EXTERNALLY ADDED DPN BY SUCCINATE IN SUBMITOCHONDRIAL PARTICLES

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It has recently been reported from several laboratories that mitochondrial DPN is reduced by succinate and that this reduction requires the addition of ATP (Azzone, Ernster and Klingenberg, 1961; Chance and Hagihara, 1960; and Klingenberg, 1960). All of these studies involved intact mitochondria and bound DPN.

In the present communication some experiments will be described which deal with the reduction of added DPN by a submitochondrial particle from beef heart muscle, ETP_H. This particle enjoys several advantages for the study of DPN reduction; it contains little or no bound DPN, and does not have a functional Krebs cycle (which excludes any reduction of DPN by substrates other than succinate).

Methods. ETP_H was prepared as described by Linnane and Ziegler (1958). DPNH formation was followed in a Beckman DK-2 spectrophotometer by the change in optical density at 340 mμ. The cuvette chamber was maintained at 38° C. Oxygen was removed from the reaction mixture by displacement with a stream of N_2. After the addition of DPN, succinate and enzyme (0.1-0.2 mg protein) to the cuvette, the mixture was preincubated for 30-60 seconds, during which time the "zero-line" was recorded. ATP, or other nucleotides which were to be tested as activators, was then added, precautions being taken to avoid mixing in of air.

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Results. In Table I, two groups of experiments are summarized. In the first, the effects of various quantities of ATP and ADP on the rate of DPN reduction are shown.

TABLE I

The Effect of Different Nucleotide Triphosphates and of ADP on the Reduction of DPN by Succinate

Expt.	Nucleotide	Addition μmoles	Rate of Reduction μmoles/min/mg protein
1	ATP	2.0	0.33
		1.0	0.29
		0.5	0.23
	ADP	1.0	0.07
2	ATP	1.0	0.16
	ITP	1.0	0.02
	GTP	1.0	0.02

Conditions: Tris, 50 μmoles; succinate, 10 μmoles; DPN, 1 μmole; 1 μmole of the nucleotides indicated in the table, final volume 1 ml, pH 7.5, temp. 38°.

The maximal rate of reduction of DPN reported here (about 0.3 μmoles/min/mg protein) coincides rather closely with the oxidation rate of this particle under phosphorylating conditions in presence of either DPNH or succinate as a substrate (Linnane and Ziegler, 1958). The possibility that the relatively slow reduction of DPN induced by the addition of ADP is due to adenylate kinase has not been excluded. Some preparations of ETP_H did not reduce DPN in presence of ADP and this inactivity of ADP in such preparations might be a reflection of adenylate kinase. As revealed by the second group of experiments in Table I, the reaction seems to be specifically linked to ATP. The rate induced by ITP and GTP was about 12% of that of ATP. The addition of UTP and CTP did not lead to the reduction of DPN.

A typical experiment is shown graphically in Figure 1 (cf. curve 1). ATP (1 μmole) was added at time zero. The limiting extent of reduction was reached

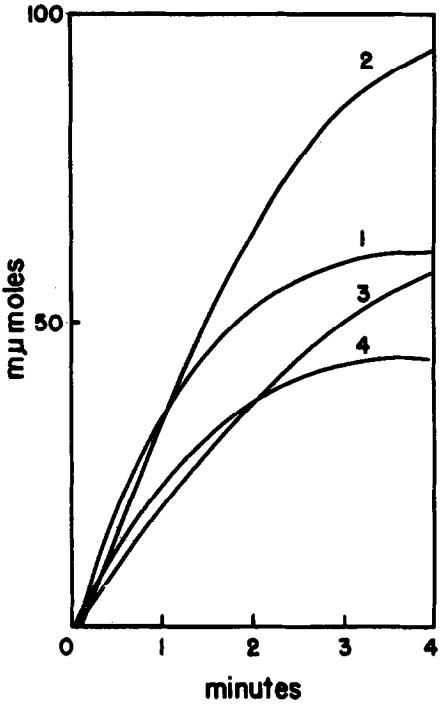

Figure 1. Reduction of DPN and DPN analogues by succinate.

Conditions: Tris, 50 μmoles; MgCl, 3 μmoles; succinate, 10 μmoles; DPN or DPN analogues, 1 μmole; ATP, 1 μmole; and enzyme, .15 mg protein. Final volume, 1 ml, pH 7.5 at 38°. Curve 1 refers to DPN, curve 2 to 3-pyridinealdehyde-deamino-DPN, curve 3 to 3-pyridinealdehyde-DPN, curve 4 to thionicotinamide-DPN. For the last analog the O.D. change was followed at 400 mμ.

after 3 minutes. With increasing amounts of ATP the extent of reduction of DPN increased correspondingly, although the quantitative relationship was not a simple one. Curves 2, 3 and 4 represent the rates of reduction of three DPN-analogues. While the initial rates of reduction of these analogues were lower than that of DPN, the amount reduced was higher in two cases. TPN was not reduced at all. On the contrary, when DPN (1 μmole) and TPN (1 μmole) were added together, the rate of change in optical density at 340 mμ was only about 65% of the rate observed with DPN alone.

In presence of inorganic phosphate the rate of reduction is markedly depressed and the inhibition is proportional to the concentration of phosphate (Table II). Although DNP inhibited the reduction of DPN by succinate

the inhibition occurred only at relatively high concentrations of DNP (cf. Table III).

TABLE II

The Effect of Inorganic Phosphate on the Rate of Reduction of DPN by Succinate

Phosphate Concentration mM	Rate of Reduction μmoles/min/mg protein	% Inhibition
0	0.14	0
5	0.094	33
10	0.057	60
20	0.042	70

Conditions: see Figure 1. Phosphate concentrations as indicated.

TABLE III

The Effect by Inhibitors on the Reduction of DPN by Succinate

Inhibitor	Concentration	% Inhibition
DNP	10^{-5}	36
"	2.10^{-5}	50
"	10^{-4}	73
Amytal	2×10^{-3} M	100
TTA[a]	10^{-3}M	100
HQNO[b]	3 μg/mg protein	24
oligomycin	1 μg/mg protein	96
"	0.5 μg/mg protein	50
"	0.25 μg/mg protein	22
antimycin A	20 μg/mg protein	89
"	12 μg/mg protein	83
"	4 μg/mg protein	50
"	2 μg/mg protein	5

[a] 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione

[b] hydroxyquinoline N-oxide

Conditions: as in Figure 1. Additions of inhibitors as indicated

The effects of a variety of other inhibitors are also listed in the Table. Since amytal inhibits the reduction of DPN by succinate, it may be inferred that the reduction is catalyzed by an electron transport component rather than by the Krebs cycle. In this connection it may be appropriate to point out that when fumarate was substituted for succinate there was no reduction of DPN whatsoever in the presence or absence of ATP.

TTA [trifluoro-1(2-thienyl)-1,2-butanadione] inhibited the reaction. This reagent has been demonstrated to be an inhibitor of succinate-CoQ reductase activity (Ziegler, 1960). HQNO or antimycin A, used in concentrations which completely block electron transport, have little effect on the rate of reduction of DPN. However, at higher concentrations of antimycin A, the reduction is inhibited and this inhibition might have to be explained in terms other than the inhibition of electron transport (e.g., an uncoupling). It is known that antimycin A at relatively high concentrations elicits an ATPase activity in intact mitochondria (Myers and Slater, 1957).

Oligomycin, shown by Lardy et al. (1958) to exert its effect on the transphosphorylation reactions of oxidative phosphorylation, inhibits the reduction of DPN almost completely at a concentration of 1 μg/mg protein but only to the extent of 22% at a concentration of 0.25 μg/mg protein. However, the yield of DPNH was increased almost 100% before the reaction came to a halt.

Discussion. The present data concerning the effects of respiratory inhibitors suggest a pathway of electrons from succinate to DPN, which can be visualized by the following scheme:

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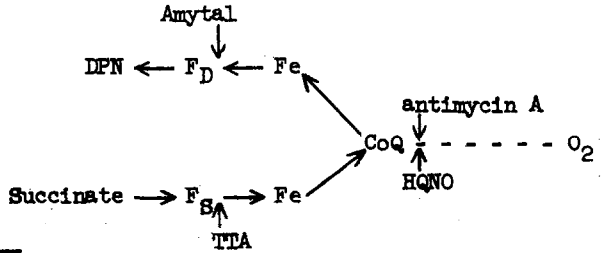

* In the scheme, F_D represents DPNH flavoprotein; F_S, succinic dehydrogenase; and Fe for non-heme iron. The above pathway agrees with scheme suggested by Hatefi, Haavik and Griffiths (1961).

The inhibition by TTA may be taken as an indication that the electrons go at least as far as CoQ. That the electrons do not proceed beyond the oxido-reduction level of CoQ is indicated by the fact that the reduction of DPN by succinate is not inhibited by antimycin A in the concentration range which is known to inhibit electron flow. The amytal inhibition definitely shows that DPN is reduced via Fe. The fact that fumarate does not reduce DPN substantiates this point.

No attempt was made to explore conditions which could increase the amount of DPN reduced for a given amount of ATP (the DPN/ATP quotient). The highest quotient observed was 0.2 μmoles DPNH per μmole ATP added. This was achieved in the presence of 0.25 μg oligomycin/mg protein.

The low value of the quotient may be a consequence of the endogenous ATPase activity of the particles. Under the conditions of our experiments, the extent of DPN reduction is determined by the level of ATP. It is of interest to point out that at low concentrations of oligomycin, the inhibition of the ATPase activity is greater than that of the DPN reduction and this differential might account for the improvement in the DPN/ATP quotient.

To summarize, ETP_H catalyzes the relatively rapid reduction of DPN by succinate in presence only of ATP. Under the particular conditions of the experiment, 0.2 μmole of DPN are reduced per mole of ATP added (no correction being made for the hydrolytic breakdown of ATP). The absence of citric cycle activity in ETP_H eliminates many objections that can be raised to the interpretation of the ATP catalyzed reduction of DPN by succinate as demonstrated in intact mitochondria. The inhibitor data permit the conclusion that only two of the four complexes of the electron transport system are implicated in this process, namely succinic-Q and DPNH-Q reductase, and that Q is the link between these two complexes. The relative ease with which the ATP catalyzed reduction of DPN by succinate can be followed in ETP_H makes it possible to study this reversal of electron transport more systematically and to exploit more fully some of the experimental advantages which this phenomenon offers.

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