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SUCCINATE-LINKED DIPHOSPHOPYRIDINE NUCLEOTIDE REDUCTION IN SUBMITOCHONDRIAL PARTICLES

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SUMMARY

1. The reduction of added DPN by succinate catalyzed by submitochondrial particles from beef heart has been studied.

2. The reduction was endergonic and required specifically the addition of ATP.

3. The reaction had a limited specificity for electron acceptors; six DPN analogues tested were reduced at the same rate or nearly the same rate, as was DPN. TPN was only reduced at a very slow rate.

4. The rate of reduction was influenced by phosphate and ADP and their effects became pronounced if added together.

5. The effect of DPNH was marked only if added in a concentration equal to or exceeding that of DPN.

6. Respiratory inhibitors acting in the flavin region of the respiratory chain blocked the reaction.

INTRODUCTION

The reversibility of the oxidative phosphorylation processes was postulated by DAVIES AND KREBS and by KORNBERG AND KREBS [2] in 1957. The first experimental evidence for a reversibility was presented by CHANCE AND HOLLUNGER[3] in 1957, who reported their studies in greater detail[4] in 1960. These first reports dealt with a reduction of intramitochondrially bound DPN by succinate *via* the respiratory chain. Intramitochondrially generated high-energy bonds from the terminal oxidation of succinate constituted the energy source for this reduction.

Simultaneously and independently KLINGENBERG *et al.*[5,6] and AZZONE *et al.*[7] had made similar findings. KLINGENBERG *et al.*[5,6] demonstrated a reduction of DPN, using α-glycerolphosphate or succinate as the electron source. ERNSTER[8] demonstrated an Amytal-sensitive reduction of acetoacetate by succinate in 1961.

All three groups have developed various aspects of these processes in further extensive studies[9–21], and a reversibility over the entire respiratory chain has been demonstrated by the oxidation of reduced cytochrome *a* by bound DPN in a system blocked with cyanide[16].

These investigations have either been dependent on a refined spectrophotometric technique for studies of shifts in the oxidation–reduction state of electron carriers on the enzyme level, or else more indirect methods have been used such as the reduction of acetoacetate, utilizing the mitochondrial dehydrogenase to trap the hydrogen from

intramitochondrially bound DPNH. In this latter case it has been possible to demonstrate reduction of DPN in "substrate" amounts with intramitochondrially generated high-energy bonds as the energy source and succinate as the electron donor[19–21].

The present paper describes some experiments in which "substrate" amounts of added DPN have been reduced by succinate in submitochondrial particles utilizing added ATP to drive the reaction.

The aspects covered in the present paper are the specificity of the source of energy, the specificity of the electron acceptor, the effects of Mg^{2+} and P_i, the effects of inhibitors of electron transport and of oxidative phosphorylation, and also the stoichiometric relationship between the amount of ATP broken down and the amount of DPNH formed. A detailed description of the procedure for the preparation of beef-heart mitochondria as well as for the submitochondrial particles is given.

A preliminary report on these findings has been published earlier[22].

MATERIAL AND METHODS

The method for the preparation of beef-heart mitochondria described here is a slight variation of the method of CRANE, GLENN AND GREEN[23], modified to suit the local conditions. The separation of heavy and light mitochondria was done according to HATEFI AND LESTER[24] and the preparation of particles, ETP_H, by the method of LINNANE AND ZIEGLER[25] with minor modifications.

Preparation of heavy beef-heart mitochondria

The beef hearts, obtained in the slaughter-house immediately after killing, were cut up radially to remove the blood. They were transported in ice containers to the laboratory cold room, where connective tissue, fat and coagulated blood was trimmed off. The pieces were cut radially and passed through a precooled electric meat grinder. The ground meat was fed into a beaker containing 0.25 M sucrose and the pH was continuously adjusted to about 7.5 with 1 M Tris. The suspension was drained on a double layer of cheese-cloth, and the material was then weighed in ice-cooled beakers into 500-g aliquots, each of which was resuspended in 1000 ml of a 0.25 M sucrose solution, 0.015 M with respect to EDTA. The suspension was stirred and saturated with nitrogen and the pH was adjusted to 7.4 with 1 M Tris. The material was further disintegrated by using an Ultra-Turrax* homogenizer for 1 min, the pH being continuously adjusted. The homogenate was centrifuged at 1550 rev./min for 20 min in 1-l containers in rotor No. 256 in an International centrifuge model PR-2. The dark red supernatant was decanted through a double layer of cheese-cloth and then centrifuged in a Servall model RC 2 centrifuge. This centrifugation was either done by continuous flow in rotor No. SS-34 at 30000 × *g*, with the system initially filled with 0.25 M sucrose, or alternatively, a rotor No. GSA with 250-ml plastic containers was used, centrifuging at 10000 × *g* for 15 min. The red brown sediment was suspended in 0.25 M sucrose, 0.01 M with respect to Tris·HCl, at pH 7.4, and homogenized in a Potter–Elvehjem glass-teflon homogenizer

The next centrifugation was done in plastic tubes in No. SS-34 rotor for 10 min

* An Ultra-Turrax TP 18/2 (Janke und Kunkel KG., Staufen in Breisgau, West Germany), is essentially a stainless-steel tube, the end of which is slit radially; a head with two knives rotates in this end at a speed of 24000 rev./min.

at 17000 × *g*. The two upper layers of light mitochondria were discarded and the lower dark layer of heavy mitochondria was re-homogenized in a glass-teflon homogenizer in sucrose–Tris solution and recentrifuged at 17000 × *g*. If light mitochondria were still remaining, they were discarded and the heavy mitochondria were finally homogenized in 0.25 M sucrose. The mitochondrial suspension was adjusted to contain 20 mg protein per ml.

The preparation has usually started with 4 beef hearts which, depending on size, gave 3000–4000 g of minced meat. The yield of heavy mitochondria varied between 1000 and 2300 mg of protein.

Preparation of submitochondrial particles (ETP_H)

The heavy beef-heart mitochondria suspended in 0.25 M sucrose were stored at −10° overnight. The suspension was thawed under the cold water tap and given a final concentration of 0.015 M Mg^{2+} and 0.001 M ATP. When saturated with nitrogen, 25-ml aliquots were exposed to sonic oscillation for 2 min in a 10 kC Raytheon Sonic Oscillator cooled with running tap-water (4–8°). A maximum effect of 1.25 A was used. The entire treated batch was centrifuged at 15000 × *g* in the Servall centrifuge, head No. SS-34, for 6 min. The supernatant fluid was then centrifuged at 100000 × *g* for 40 min in a Spinco model-L ultracentrifuge. The sediment was resuspended in about ten times its volume of 0.25 M sucrose made 0.01 M with respect to Mg^{2+}, and homogenized. This suspension, centrifuged at 100000 × *g* for 40 min gave a final sediment of particles which was homogenized and, after protein determination, adjusted to a final concentration of 20 mg of protein per ml by adding sucrose–Mg^{2+} solution.

The yield of particles was 250–400 mg protein, which corresponded to 17–22 % of the protein in the isolated mitochondria.

The uniformity of the particles was controlled by electron microscopy* using two different techniques: (a), osmium fixation, Epon embedding and sectioning; (b), the negative-staining technique according to BRENNER *et al.*[26]. Both techniques confirmed the uniformity of the particles. On the sections the particles appeared spherical and delimited by a single membrane. On the negatively stained preparations the membrane was visible as a thin line that on its outer surface was studded by tiny particles each of about 70 Å in diameter) attached by very thin filaments.

Assays

The formation of DPNH was studied by the change of absorbancy at 340 mμ in a Beckman DK-2 spectrophotometer. The cuvette chamber was maintained at 30°. The reaction mixture contained: 50 mM Tris·HCl at pH 7.5, 6 mM $MgCl_2$, 0.25 M sucrose, 10 mM succinate, 1 mM KCN and 1 mM DPN. Particles were added to a concentration of 0.15 mg of protein per ml. This mixture was preincubated for about 60 sec in order to obtain a zero-line and the reaction was started by the addition of ATP, 1 mM, giving a final volume of 3 ml. A homogeneous distribution of the reagents in the cuvette was attained by bubbling nitrogen through the reaction mixture in a short period of time.

The succinic oxidase was measured at 30° for 30 min in Warburg vessels containing 1 mM ATP, 2 mM $MgCl_2$, 12.5 mM P_i, 0.25 M sucrose, 10 mM succinate, 50 mM

* We are indebted to Dr. B. AFZELIUS for performing the electron-microscopy.

glycyl–glycin buffer, 60 mM glucose and 1.5 mg hexokinase (type 3). The amount of particles added corresponded to 1 mg of protein and the final volume was 1 ml. The centre well contained 0.1 ml of 2 M KOH.

Protein was determined by the method of GORNALL *et al.*[27] and phosphate according to Martin and Doty as described by LINDBERG AND ERNSTER[28].

RESULTS

Phosphorylating submitochondrial particles were capable of reducing added DPN with succinate as the electron donor. The reduction was endergonic and required the addition of ATP. In the experiment illustrated in Fig. 1, the particles were incubated with succinate and DPN in the presence of cyanide. The addition of ATP at zero time initiated the reduction of DPN.

Fumarate, added in the same concentrations as succinate, and in the presence or absence of ATP, did not result in any reduction of DPN. This rules out the possibility of a reduction by means of DPN-coupled dehydrogenases, and classifies the reduction as a "reversal" of terminal electron transport. Over the range used in the assays the rate of DPN-reduction was proportional to enzyme concentration (Fig. 2).

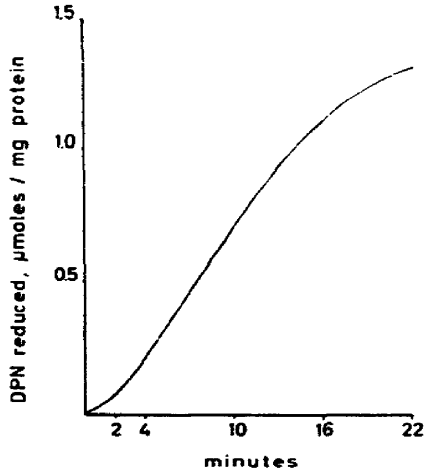

Fig. 1. Reduction of DPN by succinate on the addition of ATP. The cuvette contained in a final volume of 3 ml, 50 mM Tris·HCl buffer (pH 7.5), 6 mM $MgCl_2$, 0.25 M sucrose, 10 mM sodiumsuccinate, 1 mM DPN and 1 mM KCN. Enzyme particles were added corresponding to a protein concentration of 0.15 mg/ml, the baseline was recorded for about 60 sec before the addition of 1 mM ATP. The figure shows the DPN-reduction upon the addition of ATP recorded as an increase in absorption at a wavelength of 340 mµ. The temperature of the incubation was 30°.

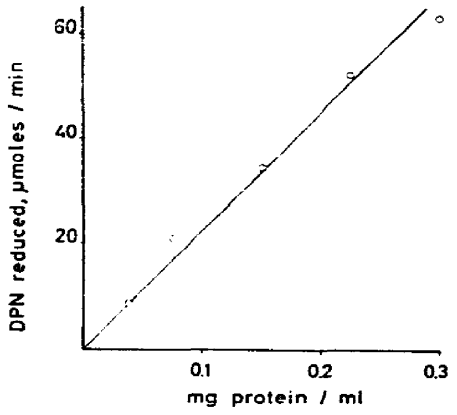

Fig. 2. The effect of varying concentrations of enzyme particles on the rate of reduction of DPN. Experimental conditions were the same as in Fig. 1. Enzyme concentrations as indicated in the figure.

The reduction was completely dependent on the addition of Mg^{2+}. Maximal stimulation was obtained at about 5–6 mM of Mg^{2+}, as shown in Table Ia. The addition of EDTA inhibited the reduction (Table Ib) to an extent greater than could be expected from the binding of the added Mg^{2+}. This is taken as an indication of the involvement of other sites with functional bivalent metal ions than the site depending on the added Mg^{2+}.

Specificity for ATP

The reduction was dependent on ATP. No other nucleotide tested was in the present system capable to supply energy for the reaction (Table IIa). The reduction obtained upon the addition of ADP was most likely due to an adenylate kinase

TABLE Ia

THE EFFECT OF Mg^{2+} ON THE REDUCTION OF DPN

Experimental conditions as in Fig. 1. $MgCl_2$ concentrations as indicated in the table.

Mg^{2+} (mM)	*DPN reduced (mμmoles/ min/mg of protein*
0	0
1	55
3	63
5	79
6	79
9	70

TABLE Ib

THE EFFECT OF EDTA ON THE REDUCTION OF DPN

Experimental conditions as in Fig. 1. EDTA concentration as indicated in the table.

EDTA (mM)	*Inhibition (%)*
1	34
2.5	49
5	71
7.5	100

activity in the particle. In the presence of hexokinase and glucose to trap ATP, ADP was no longer able to drive the reduction. The addition of phosphoenolpyruvate and pyruvate kinase had no effect on the rate of reduction. This was somewhat unexpected in view of the finding of PENEFSKY *et al.*[29,30] that an increase in the ATPase rate of a similar preparation occurred when phosphoenolpyruvate and pyruvate kinase were used as a feed-in system of high-energy phosphate. These authors have also reported a potent ITPase activity in their purified preparation. The particles used in the present investigation also possessed an ITPase activity, which was about half of that of the ATPase.

ADP has little, or no, effect on the reduction of DPN if added together with the ATP. If added in equimolar concentration no inhibition could be observed, compared with an inhibition of about 15% when the molar ratio of ATP to ADP was 1/5 (Table IIa).

Effect of phosphate

Phosphate had a limited inhibitory effect on the reaction when added alone. It started to inhibit in concentrations above 10 mM (Table IIb), However, if ADP was

present, the P_i effect was more pronounced. The inhibition by phosphate reported earlier[22] might have been due to the presence of a higher unspecific ATPase activity, which brought up the ADP level. The earlier experiments reported by Löw *et al.*[22] showed a linear reduction during a shorter time interval and a lower efficiency as expressed by the ratio of DPN-reduced to ATP added.

TABLE IIa

THE EFFECT OF VARIOUS NUCLEOTIDES ON THE REDUCTION OF DPN

Experimental conditions as in Fig. 1. ATP is substituted by other nucleotides where indicated. Hexokinase and pyruvate kinase where indicated, were added in great excess.

	Nucleotide addition	*Concentration (mM)*	*DPN reduced (mμmoles/min/mg of protein)*
Expt. 1	ATP	1.0	46
	ATP	2.0	51
	CTP	1.0	0
	GTP	1.0	0
	ITP	1.0	0
	AMP	1.0	0
	ADP	1.0	23
	ADP + hexokinase	1.0	0
Expt. 2	ATP	0.07	14
	ATP	0.10	23
	ATP	0.17	42
	ATP	1.0	70
	ATP + phosphoenol pyruvate + pyruvate kinase	1.0 + 1.0	63
Expt. 3	ATP	1.0	69
	ATP + ADP	1.0 + 1.0	67
	ATP	0.2	46
	ATP + ADP	0.2 + 1.0	39
	ADP	1.0	23

TABLE IIb

THE EFFECT OF INORGANIC PHOSPHATE ON THE REDUCTION OF DPN

Experimental conditions as in Fig. 1. Where indicated, ADP was added together with the ATP to a final concentration of 1 mM. P_i concentrations as indicated in the table.

		DPN reduced	
	P_i (mM)	*(mμmoles/min/mg of protein)*	*+ ADP (mμmoles/min/mg of protein*
Expt. 1	—	79	80
	0.33	79	76
	1.66	69	39
	3.33	72	25
Expt. 2	—	68	—
	1	73	—
	2	79	—
	5	79	—
	10	51	—

The specificity of the electron acceptor

The specificity for DPN as an electron acceptor is relatively small (Table IIIa). Six DPN analogues were tested. Four of the analogues were substituted in different ways in the 3-position of the pyridine ring, one had the adenine moiety converted to inosine, and two were altered both in the adenine moiety and in the 3-position of the pyridine ring. They were all less good electron acceptors than DPN, but in no case was the rate of reduction lower than about 60% of that of DPN.

TPN, on the other hand, was only reduced with a rate of about 15% of that of DPN, and if present together with DPN, caused a lower rate of reduction as measured by the increase in absorption at 340 mμ (Table IIIb). Under the present conditions this inhibition was not greatly influenced by change of the DPN/TPN ratio.

The addition of DPNH together with DPN did not drastically influence the rate of reduction. At equimolar concentrations of DPNH and DPN the rate of reduction was only 22% lower than in the control (Table IIIb).

TABLE IIIa

REDUCTION OF DPN AND SOME DPN ANALOGUES BY SUCCINATE

Experimental conditions as in Fig. 1, with substitution of DPN by the various analogues tested. The analogues were purchased from Pabst Laboratories, Milwaukee, Wis., and the spectral properties of the analogues were quoted from their circular OR-18.

	Absorption change at wavelength (mμ)	*Extinction coefficient used (mM)*	*Acceptor reduced*	
			Expt. 1 (mμmoles/min/mg of protein)	*Expt. 2 (mμmoles/min/mg of protein)*
DPN	340	6.2	69	65
3-Acetylpyridine-DPN	363	9.1	61	61
3-Pyridinealdehyde-DPN	358	9.3	41	37
Thionicotinamide-DPN	395	11.3	56	57
Deamino-DPN	338	6.2	54	53
3-Acetylpyridine-deamino-DPN	361	9.0	43	44
3-Pyridinealdehyde-deamino-DPN	356	9.4	42	43

TABLE IIIb

THE EFFECT OF DPNH AND TPN ON THE REDUCTION OF DPN

Conditions as in Fig. 1.

	DPN (mM)	*TPN (mM)*	*DPNH (mM)*	*Acceptor reduced (mμmoles/min/mg of protein)*
Expt. 1	1.0	—	—	51
	2.0	—	—	51
	1.0	1.0	—	42
	—	1.0	—	7
Expt. 2	0.2	—	—	59
	0.2	—	0.1	54.5
	0.2	—	0.2	46
	0.2	—	0.3	28
	0.1	—	—	54.5
	0.1	—	0.1	54.5

Relation between DPNH formed and ATP added

The reduction of DPN proceeded with a linear rate for about 10–15 min under the present conditions, at which time it started to level off and finally reached equilibrium (*cf.* Fig. 1). The ATP remaining was determined after the reduction had come to a halt. 2.8 μmoles of the initially added 3.0 μmoles of ATP had disappeared. 2.84 μmoles of phosphate were liberated during the same experiment. The remaining 0.2 μmole (0.07 mM) was only able to drive the reduction at a relatively low rate, provided the ADP and P_i concentrations were low (as in Table IIa). Further addition of ATP resulted in a further reduction of DPN although at a lower rate and to a smaller extent. Upon a second addition the reaction leveled off well before the ATP was used up. The extent of reduction seemed to be greatly influenced by the concentrations of inorganic phosphate and ADP (*cf.* Table IIb). Again the ratio of DPNH to DPN seemed to play a small role.

At equilibrium in the above experiment 2.8 μmoles of ATP corresponded to 0.6 μmole of DPN reduced, giving an ATP/DPNH quotient of 4.7/1.

The ATP/DPNH quotient was also measured under somewhat different conditions. The incubation was stopped by the addition of perchloric acid during the linear portion of the reaction. As seen in the Table IV the ATP/DPNH quotient under these conditions varied between 3.2 and 3.5. The ATP which was used up in the reaction was assumed to be equal to the P_i liberated. In the same table the P_i liberated under the same conditions but when no DPN was added, is also given. In all three experiments the presence of DPN increased the amount of P_i liberated. If this increase of P_i caused by DPN is taken as the basis for the calculation of the ATP/DPNH quotient, values from 0.7 to 0.3 are obtained. It seems likely that an estimation of the ATP/DPNH quotient based on the gross break-down of ATP during the reduction of DPN involves an overestimation of the ATP requirement due to the unspecific ATPase activity. On the other hand, if the increase in the liberation of P_i is taken as a basis for the estimation, the quotient is probably underestimated, due to a competition between DPN reduction and non-specific hydrolytic splitting for the primary high-energy intermediate. Thus the present data can only indicate that the quotient is lower than 3.4 and higher than 0.3.

TABLE IV

DETERMINATION OF THE ATP/DPNH QUOTIENT

Experimental conditions were the same as those in Fig. 1 with the exception that the reference cuvette contained a complete reaction mixture minus DPN. The P_i liberated was determined by taking out an aliquot for analysis simultaneously from each of the two cuvettes. — DPN refers to the reference cuvette, + DPN to the sample cuvette. ΔP_i refers to the stimulation of phosphate liberation caused by DPN. P_i/DPNH refers to total amout of DPN reduced during the same period. ΔP_i/DPNH refers to the increase in phosphate liberation caused by the presence of DPN during the incubation period over the amount of DPN reduced during the same period.

	Enzyme protein (mg/ml)	*DPNH formed (μmoles)*	*P_i liberated*		*ΔP_i (μmoles)*	*P_i/DPNH*	*ΔP_i/DPNH*
			— DPN (μmoles)	*+ DPN (μmoles)*			
Expt. 1	0.45	0.24	0.68	0.81	0.13	3.24	0.54
Expt. 2	0.45	0.26	0.66	0.83	0.17	3.22	0.65
Expt. 3	0.67	0.27	0.85	0.92	0.07	3.45	0.26

Effect of inhibitors

To elucidate the electron path from succinate to DPN, a series of inhibitors of electron transport were tested. As seen in Table V, inhibitors known to act in the flavin region inhibited the reduction of DPN. 4,4,4-Trifluoro-1-(2-thienyl)-1,3-butanedione, reported to act as an inhibitor of the succinic-CoQ reductase[31] inhibited the reaction. Amytal, known to act in the diaphorase flavin region[32], was a potent inhibitor of the reduction in concentrations even lower than needed for complete inhibition of the DPNH oxidase. Rotenone, which like Amytal is reported to inhibit the DPNH oxidase[33,34] by titrating a site in the flavoenzyme[35] also proved to be a powerful inhibitor of the reduction of DPN (Table V).

TABLE V

EFFECTS OF AMYTAL, ROTENONE AND 4,4,4-TRIFLUORO-1-(2-THIENYL)-1,3-BUTANEDIONE ON THE REDUCTION OF DPN

Conditions were as in Fig. 1. Rotenone and 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione were each added dissolved in 2 μl of ethanol.

Inhibitor	*Concentration (mM)*	*Inhibition (%)*
4,4,4-Trifluoro-1-(2-thienyl)-1,3-butanedione	0.027	37
	0.054	45
	0.11	74
	0.22	83
Amytal	0.12	13
	0.24	32
	0.48	87
	0.72	93
	(mμmoles/mg of protein)	
Rotenone	0.014	31
	0.026	45
	0.055	68
	0.11	87
	0.22	100

For antimycin A[36] and 2*N*-nonyl-hydroxyquinoline-*N*-oxide[37], inhibitors of electron transport acting in the cytochrome region, the picture is more complex. As demonstrated in Table VI both antimycin A and 2*N*-nonyl-hydroxyquinoline-*N*-oxide, when in high concentration, inhibited the reduction of DPN. In the case of antimycin A, however, this inhibition occurred at levels which exceeded, by ten- to a hundred-fold, the level needed to give the same inhibition of the oxidation of succinate when oxygen was used as an electron acceptor (Table VII). It has been argued that "it is not to be expected that the quantitative effect of inhibitors of reversed electron transfer would be identical with that on forward electron transfer since the rates of electron flow differ by about ten-fold"[9]. During this investigation the rate of oxidation of succinate using DPN as the electron acceptor was between 0.05 and 0.12 μmole/min/mg of protein; the same rate using oxygen as the electron acceptor was 0.20–0.30 μatom/min/mg of protein. The antimycin A inhibition was also tested under conditions where the rate of electron flow was made similar by partially blocking the

succinic dehydrogenase with malonate. Thus, when the rate of oxidation of succinate was brought down to 0.10 μmole/min mg of protein, the amount of antimycin A needed to inhibit the reaction was the same as needed when the enzyme worked at full capacity (Table VII).

TABLE VI

EFFECTS OF ANTIMYCIN A AND 2*N*-NONYL-HYDROXYQUINOLINE-*N*-OXIDE ON THE REDUCTION OF DPN

Conditions were as in Fig. 1. The inhibitors were each added dissolved in 2 μl of ethanol.

Inhibitor	*Concentration (μg/mg of protein)*	*Inhibition (%)*
Antimycin A	1	13
	2	18
	4	37
	12	63
2*N*-Nonyl-hydroxyquinoline-*N*-oxide	0.9	8
	1.9	27
	2.5	43
	3.8	63
	5.1	84
	7.5	100

Although it might be the "simplest" explanation to attribute the inhibition of antimycin A to its classical effect[9], we would prefer to regard it as a secondary uncoupling effect resulting in an ATPase activity as described by MYERS AND SLATER[38].

2*N*-Nonyl-hydroxyquinoline-*N*-oxide in high concentrations also inhibited the reduction of DPN. To block the oxidation of succinate with DPN or oxygen as electron acceptors respectively, required ten times less 2*N*-nonyl-hydroxyquinoline-*N*-oxide in the later case. We are inclined to believe that the argument used for the mode of

TABLE VII

INHIBITION OF SUCCINATE OXIDATION BY ANTIMYCIN A AND 2 *N*-NONYL-HYDROXYQUINOLINE-*N*-OXIDE

Experimental conditions are given in METHODS. The inhibitors were dissolved in ethanol and added in volumes of 2 μl each.

Inhibitor	*Concentration (μg/mg of protein)*	*Inhibition*	
		— (%)	+ *malonate* (%)
Antimycin A	0.05	6.9	0
	0.1	34.6	18
	0.12	41.4	35
	0.13	82.7	80
	0.15	100	100
2*N*-Nonyl-hydroxyquinoline-*N*-oxide	0.15	2.8	19
	0.2	8.2	22
	0.3	44.3	32
	0.4	67.2	66
	0.6	86.9	68
	0.65	90.2	83

action of antimycin A above might also be applied to the action of 2*N*-nonyl-hydroxyquinoline on the succinate-linked reduction of DPN.

Agents blocking transphosphorylation such as oligomycin, or which uncouple transphosphorylation from electron transport such as DNP or dicoumarol were potent inhibitors of the reduction of DPN (Table VIII). Oligomycin[39,40] gave a 50% inhibition at about 0.07 μg/mg of protein. DNP and dicoumarol gave a 50% inhibition at $3 \cdot 10^{-6}$ M and $5 \cdot 10^{-7}$ M respectively, indicating that any interference with the energy supply had a severe consequence on the reaction.

TABLE VIII

EFFECTS OF DNP, DICOUMAROL AND OLIGOMYCIN ON THE REDUCTION OF DPN

Conditions as in Fig. 1. Oligomycin and dicoumarol were dissolved in ethanol and added in volumes of 2 μl each.

Inhibitor	*Concentration (μg/mg of protein)*	*Inhibition (%)*
Oligomycin	0.043	29
	0.063	44
	0.08	69
	0.13	93
	(mM)	
DNP	10^{-3}	17
	$2 \cdot 10^{-3}$	40
	$3 \cdot 10^{-3}$	51
	$5 \cdot 10^{-3}$	72
	$6 \cdot 10^{-3}$	90
Dicoumarol	10^{-4}	13
	$2.5 \cdot 10^{-4}$	22
	$7 \cdot 10^{-4}$	54
	10^{-3}	68

DISCUSSION

The results of the present investigation agree with earlier findings of a reversal of oxidative phosphorylation. The simplicity of the system: the reduction of substrate amounts of added DPN by succinate made energetically possible by the addition of ATP, dismisses some of the doubts that have been raised[41] about the significance of the involvement of the respiratory chain in the reduction of DPN by succinate.

Substantial oxidation of succinate by DPN has in earlier reports been coupled to a trapping of hydrogen by *e.g.* acetoacetate[19–21] thus involving a mitochondrial dehydrogenase. KREBS AND EGGLESTON[41] have argued that most of the acetoacetate reduction involves only the mitochondrial dehydrogenases, and that the reduction *via* the respiratory chain is of little significance. They point out that the reduction of DPN *via* the respiratory chain might be of significance only during short-time incubations (1–2 min) whereas, in their experiments, with an incubation time of 30–60 min, this pathway was of minor importance.

In the present investigation an enzymic preparation has been used which is to a large extent deficient in mitochondrial dehydrogenases. Thus the addition of fumarate

does not cause reduction of DPN under any circumstances, showing that, in these experiments, the only possible pathway for electron transfer is *via* the respiratory chain. The reduction is linear with time for approx. 15 min and proceeds further in a non-linear fashion for periods up to 30 min under the conditions used. It is therefore difficult to see how the objections raised by KREBS AND EGGLESTON[41] against the involvement of the respiratory chain in the reduction of DPN by succinate could apply under the present conditions.

The conditions used in this investigations also seem to rule out another objection which has been raised in connection with experiments which show only the reduction of intramitochondrially bound DPN. It has been argued[42] that it is difficult to distinguish between the possibilities of whether succinate, as such, is the source of electrons, or whether succinate activates some internal source of electrons which in turn reduce DPN. The amount of DPN reduced in the present experiments by far exceeds the amount of endogenous substrate present.

In one respect the present findings are at variance with those of other workers. It has been reported by others[10,16] that when externally added ATP is used to drive the reaction, added Mg^{2+} is found to be inhibitory, and reduction is facilitated by the addition of EDTA. When the reduction is catalyzed by submitochondrial heart particles, as in the present case, Mg^{2+} is a prerequisite for the reduction (Table Ia). EDTA (Table Ib) strongly inhibits the reaction, even to a greater extent than could be expected from its binding of the added Mg^{2+}. This may mean that the previously reported effects of Mg^{2+} and EDTA are due to an interference of Mg^{2+} on some transfer mechanisms[43] connected with the intact mitochondrial membrane structure which are not present in submitochondrial preparations of this type.

The investigation of the action of respiratory inhibitors in the present system gives an idea about the electron pathway which may be involved. Inhibitors acting on the flavin level such as Amytal and Rotenone[32–35], which block the electron transport through the diaphorase flavin, are inhibitory; this is in agreement with what has been reported by others. 4,4,4-Trifluoro-1-(2-thienyl)-1,3-butanedione, acting on the non-heme iron constituent of the succinic–CoQ reductase, also inhibits the reduction[31]. Although the specificity of this inhibitor might be questioned, its inhibitory effect excludes the possibility of a direct transfer of the electrons from the succinic dehydrogenase flavin to the diaphorase flavin.

The inhibition caused by antimycin A reported in this paper is not attributed to the classical ability of antimycin A to block the electron transport in the cytochrome region between cytochrome *b* and cytochrome *c*, but it is attributed to its capacity at higher concentrations to affect the coupling system. The concentration needed for 50% inhibition of succinate oxidation with DPN as the electron acceptor is about a hundred times greater than that needed to give the same inhibition when oxygen is used as acceptor.

The present interpretation conforms with that of KLINGENBERG AND SCHOLLMEYER[13] and also with that of ERNSTER *et al.*[21]. It is at variance with the interpretation of CHANCE AND HOLLUNGER[9], who reported a decrease in the rate of reduction of intramitochondrially bound DPN with approximately the same concentrations of antimycin A as used here. KLINGENBERG AND SCHOLLMEYER found that, upon the addition of antimycin A, the endogenous source of energy could no longer be used for driving the reduction of bound DPN. Their source of electrons was glycerol 1-phos-

phate. Added ATP, however, could be used as the energy source to drive the reduction of DPN in the presence of antimycin A also. ERNSTER *et al.*[21], who have been using internally generated high-energy intermediates to drive the reduction of acetoacetate from succinate, also demonstrated an antimycin A insensitivity in their system. Since they were dependent on the terminal electron-transport system for the supply of energy, antimycin A alone could not be used to demonstrate whether the side of inhibition of this drug was involved or not. However, by the addition of ferricyanide together with antimycin A, they were able to bring about the reduction of acetoacetate with energy supplied by the antimycin A-insensitive oxidation of ferricyanide thus demonstrating the antimycin A insensitivity of the pathway from succinate to acetoacetate.

It has been suggested that the ATPase activity of structurally disrupted mitochondria is part of the enzymic machinery of oxidative phosphorylation[44]. This view is further stressed by the ATP-dependent DPN reduction. The relatively weak, and varying, stimulation by DPN of the rate of liberation of P_i incidates, as suggested earlier[45], that hydrolysis of the high-energy linkage might occur somewhere along the line to the point of the functional energy utilization.

From the present data it might be possible to go one step further in defining the relation between the oxidative machinery and the nucleoside triphosphatase activity. The finding that ATP is the only nucleoside triphosphate tested which was able to accomplish the reduction of DPN indicates that only the ATP-specific nucleoside triphosphatase activity might be regarded as a part of the coupling system. This is in accordance with the finding that only ADP may be used as the phosphate acceptor for the electron-coupled phosphorylation in submitochondrial particles as well as in intact mitochondria.

The present system opens possibilities to prove or disprove the existence of a high-energy intermediate of DPNH ~ I or DPN ~ I type[46]. Although the present investigation adds no convincing evidence as to whether the primary high-energy bond is connected to the pyridine nucleotide side or to the flavin side, the lack of specificity both in the substituents of the pyridine ring and in the adenine part of DPN demonstrated here, argues against the concept of the formation of a high-energy bond in connection with pyridine nucleotide.

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