On the Mechanism of Oxidative Phosphorylation. VII. The Energy-Requiring Reduction of Pyridine Nucleotide by Succinate and the Energy-Yielding Oxidation of Reduced Pyridine Nucleotide by Fumarate*

D. R. SANADI AND ARVAN L. FLUHARTY

From the Gerontology Branch, National Heart Institute, National Institutes of Health, and the Baltimore City Hospitals, Baltimore, Maryland

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Phosphorylating electron transport particles prepared by the sonic disruption of beef heart mitochondria catalyze the adenosine triphosphate-dependent reduction of nicotinamide adenine dinucleotide (NAD) by succinate and the fumarate-dependent oxidation of reduced nicotinamide adenine dinucleotide (NADH). Coupled phosphate esterification has been observed in the latter reaction. Both reactions are inhibited by malonate, Amytal, and *high* levels of antimycin. Dicumarol, 2,4-dinitrophenol, and carbonyl cyanide *p*-chlorophenylhydrazone inhibit only the energy-requiring reduction of NAD by succinate. The results clearly confirm the reversibility of oxidative phosphorylation in the NADH region of the respiratory chain.

In recent years a number of laboratories have provided evidence that the normal flow of electrons from NADH to oxygen which is coupled to ATP synthesis could be reversed by regulating the conditions so that energy from ATP or an intramitochondrial "energy-rich" intermediate is utilized (Chance and Hollunger, 1960, 1961a,b; Chance and Hagihara, 1960a, b; Klingenberg and Slenczka, 1959; Klingenberg and Schollmeyer, 1961; Azzone *et al.*, 1960; Chance and Fugman, 1961). A direct demonstration of the reversal of oxidative phosphorylation was achieved by Löw *et al.* (1961), who found that phosphorylating electron transport particles (ETP_H) from beef heart mitochondria catalyzed the reduction of added NAD by succinate in the presence of ATP (Reaction 1). The reaction provides a convenient spectrophotometric assay for the reactions associated with oxidative phosphorylation in the NADH region of the respiratory

$$\text{succinate} + NAD^+ + ATP \rightleftharpoons \text{fumarate} + NADH + H^+ + ADP + P_i \quad (1)$$

chain. However, it is not possible to distinguish between the effects of uncoupling agents and electron transport inhibitors in this system, since both lead to inhibition of NAD reduction. We have found that

$$NADH + H^+ + \text{fumarate} \longrightarrow NAD^+ + \text{succinate} \quad (2)$$

submitochondrial particles also catalyze the thermodynamically favorable oxidation of NADH by fumarate (Reaction 2), a reaction previously observed by Slater (1950). Evidence is presented here that the respiratory carriers involved in the two reactions are probably identical. The two systems together permit a reasonably satisfactory distinction between uncoupling agents and respiratory inhibitors and provide assays for following further fractionation of the oxidative phosphorylation system. Preliminary reports of this work have appeared previously (Fluharty and Sanadi, 1962; Sanadi *et al.*, 1962).

EXPERIMENTAL PROCEDURE

The mitochondrial particles (ETP_H) were prepared by a procedure which included several minor modifications of the method of Linnane and Ziegler (1958). Diced beef heart was homogenized in 125-g lots with 250 ml of 0.25 M sucrose containing 0.016 M Tris[1] base in a Waring Blendor for 30 seconds. The homogenate from six such batches was mixed with 1 liter of 0.25 M sucrose, adjusted to *p*H 7.6 with Tris base and centrifuged at 1100 × *g* for 10 minutes. The sediment was resuspended in 1 liter of 0.25 M sucrose, rehomogenized in the Waring Blendor for 10 seconds, and again centrifuged as before. The combined supernatant liquid from the two previous steps was centrifuged at 15,000 × *g* for 10 minutes. The mitochondrial sediment was washed by suspension in approximately 250 ml of 0.25 M sucrose containing 1 mM Tris acetate at *p*H 7.8 and recentrifuged at 15,000 × *g*. The supernatant liquid was discarded, and the loosely packed portion of the sediment (light mitochondria) was sloughed off by swirling with water. The residual tightly packed material (containing 1 to 2 g of protein) was suspended in 150 ml distilled water and subjected to sonic oscillation for 45 seconds in 25-ml batches in the cooled 20 kc "Biosonik" of the Will Corporation. The disrupted mitochondrial suspension was centrifuged at 25,000 × *g* for 10 minutes, and the supernatant fluid containing fragments of disrupted mitochondria was recentrifuged at 100,000 × *g* for 30 minutes. The sediment (yield of approximately 500–600 mg protein) was suspended in 0.25 M sucrose and used as such or stored frozen at a protein concentration of 10 to 20 mg per ml.

For the assay of Reaction (1), the medium contained 0.05 M Tris chloride[2] buffer at *p*H 7.5, 0.1 mM EDTA, 3.3 mM $MgCl_2$, 6.7 mM succinate, 1 mM NAD, 1 mM KCN, and 0.75 mg ETP_H protein in 2.9 ml. Immediately after the addition of cyanide and ETP_H, the mixture was incubated at 30° for 3 minutes. The reaction was initiated by the addition of 0.1 ml of 0.06 M ATP, and NAD reduction was followed by measuring the absorbancy at 340 mμ every 2 minutes, the first reading being taken 2 minutes after the addition of ATP.

Reaction (2) was followed as above except that the medium contained no succinate, and 0.56 μmole of NADH was present instead of NAD. After incubation at 30° for 3 minutes, the reaction was started by the

* For paper VI of this series see Fluharty and Sanadi (1963).

[1] The following abbreviations are used: Tris for tris-(hydroxymethyl)aminomethane; EDTA for ethylenediaminetetraacetate; ETP_H for submitochondrial particles prepared by methods described here and slightly different from the original ETP_H of Linnane and Ziegler (1958); TTB for 4,4,4-trifluoro-1(2-thienyl)-1,3-butanedione.

[2] More recent work has shown that roughly 25% higher activity is obtained if Tris sulfate is used in the assay instead of Tris chloride.

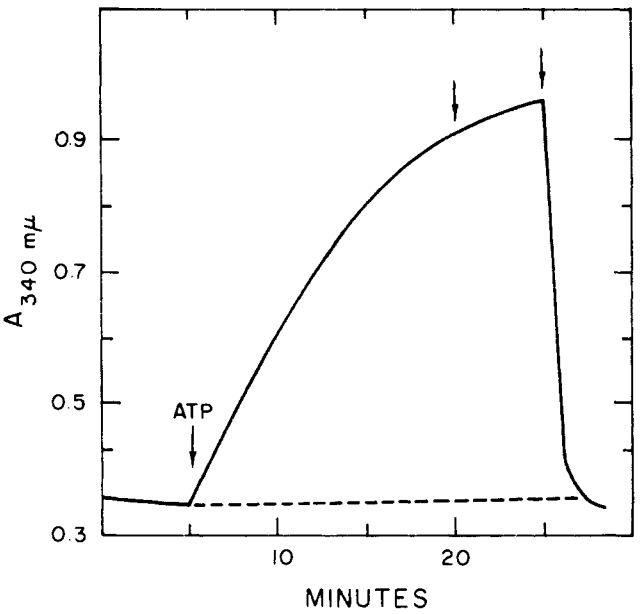

FIG. 1.—The ATP-dependent reduction of NAD by succinate. The experimental conditions were as in the text except 0.01 M phosphate buffer was used instead of Tris. ATP was added at 5 min., 0.1 ml of 0.5 M acetaldehyde at 20 min., and 5 μg crystalline alcohol dehydrogenase at 25 min. The broken line shows the activity in the absence of added ATP.

addition of 0.1 ml of 0.1 M fumarate. A control reaction containing no fumarate was always carried out concurrently to correct for fumarate-independent NADH oxidation.

The experiments on the aerobic oxidation of NADH or succinate, for comparison of the effects of antimycin, were carried out under the conditions described for the assay of Reaction (1) except that no cyanide, fumarate, NAD, or ATP were present (see Results). The oxygen concentration in the medium was measured polarographically (Chance and Williams, 1955) with a stationary platinum microelectrode and rotation of the reaction vessel at 60 rpm with a synchronous motor. The rate of reduction of cytochrome *c* (0.1 mg) by succinate was also measured in a medium identical to that used for assay of Reaction (1) except that NAD and ATP were not present.

The separation of C^{14}-succinate and C^{14}-fumarate from the reaction products was carried out by a modification of the ion-exchange method described by Busch *et al.* (1952). After completion of the reaction, the incubation medium was heated in a bath at 100° for 10 minutes and the precipitated protein was separated by centrifugation. The residue was washed, and the combined washings and first supernatant fraction were applied to a 0.6 × 6 cm column of Dowex 1 (formate), X8, 100–200 mesh. The column was then washed with 20 ml of water followed by 20 ml of 2 N formic acid. The eluate was collected in 5-ml fractions, and the first two fractions, which contained roughly 95% of the succinic acid, were handled as described later. The column was next washed with 6 N formic acid and 5-ml fractions were again collected. Roughly 90% of the fumarate appeared in the first fraction, which was counted in a thin-window G.M. counter after evaporation. The succinic acid fractions were evaporated to dryness *in vacuo*, 2 mg of carrier succinic acid was added, and the material was sublimed *in vacuo* at 120°. An additional mg of succinic acid (in solution) was added to the residue and again sublimed after drying. The procedure was repeated a third time to ensure complete recovery of labeled succinic acid. The combined sublimates were plated and counted as above. The purification by sublimation was essential, since malic and succinic acids are not separated on the Dowex column under the above conditions.

Antimycin A was purchased from Wisconsin Alumni Research Foundation, TTB from Eastman Kodak Co., and hexokinase (Type III) from Sigma Chemical Co. Carbonyl cyanide *p*-chlorophenylhydrazone was a gift from Dr. P. G. Heytler, and oligomycin was kindly provided by Dr. H. A. Lardy. Other methods have been described elsewhere (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1961).

RESULTS

Reaction Properties.—The course of the ATP-dependent reduction of NAD by succinate in a typical experiment is illustrated in Figure 1. The reaction rate calculated from the 2 to 4 minute change in absorbancy under standard conditions was generally in the range of 40 to 100 mμmoles of NAD reduced per minute per mg protein. The rate declined with time with the more active preparations. Recently, it has been found that the activity is stimulated by the addition of the supernatant extract derived from the 100,000 × *g* centrifugation of the sonically disrupted mitochondria (Sanadi *et al.*, 1962). The above variability in the activity may be due to retention in the particles of different amounts of the activator. The change in absorbancy over the 8-minute period was less than 0.01 in the absence of ATP, NAD, or succinate. The maximum activity observed by Löw *et al.* (1961), employing an ETP_H prepared under somewhat different conditions, was reported to be at least 3-fold greater at 38°. Addition of crystalline yeast alcohol dehydrogenase and acetaldehyde after the reaction had proceeded for some time resulted in rapid decrease of the absorbancy to the original value, indicating that the absorbancy change at 340 mμ was a result of NADH formation. Under our experimental conditions, Reaction (1) ceased when approximately one third of the added NAD had been reduced. It was not possible to establish the stoichiometry of the reaction from these data, since the particles had high ATPase activity (Löw *et al.*, 1961).

A typical assay for Reaction (2) is shown in Figure 2. The activity in the absence of fumarate was usually 20 to 30% of the rate obtained with the complete system. All data presented here for Reaction (2) have been corrected by subtraction for this fumarate-independent, cyanide-insensitive NADH oxidation, although the validity of such a correction may be questioned. The usual activity of our preparations was 20 to 30 mμmoles NADH oxidized per minute per mg protein, which is over ten times the activity observed by Slater (1950) with the Keilin-Hartree type of heart muscle particles.

In order to prevent aerobic oxidation of NADH and succinate through the cytochrome system, it was necessary to carry out Reactions (1) and (2) under nitrogen or in the presence of 1 mM cyanide or 0.3 mM sodium sulfide for inhibition of the cytochrome oxidase. Similar activities were obtained with each of these different methods for prevention of terminal oxidation.

The activity of ETP_H in Reaction (1) was lost rapidly when the particles were frozen as a suspension in water. When the particles were frozen in 0.25 M sucrose, the activity was unchanged for 6 days and, subsequently, half of the activity was lost in approximately 20 days. On the other hand, the activity of ETP_H frozen in sucrose solution was unaltered in the fumarate-dependent NADH oxidation (Reaction 2) even after 2 months.

The activity of a similar suspension in Reaction (1) was reduced after 1 and 2 days to 24% and 14%, respectively, of the initial level when it was maintained at 4°. The residual activity in Reaction (2) was 75% and 65% under these conditions of storage after the same respective intervals.

Mg^{++} was an absolute requirement for the ATP-dependent reduction of NAD by succinate (Table I).

TABLE I

REQUIREMENT FOR Mg^{++} FOR THE ATP-DEPENDENT REDUCTION OF NAD BY SUCCINATE

Experimental conditions are described in the text.

Conditions	$\Delta A_{340\ m\mu}$/8 min.	
	Reaction 1	Reaction 2
Complete system	0.325	0.240
No $MgCl_2$	0.005	0.244
No EDTA	0.340	0.175

This Mg^{++} dependence for activity in beef heart particles has been confirmed by Hommes (1962) in Chance's laboratory. Reaction (2), however, was unaffected by Mg^{++} (Table I). EDTA consistently activated Reaction (2) slightly but did not affect Reaction (1). Mg^{++} and EDTA were present in both assays for the sake of unformity in the comparison of the effect of inhibitors. For the same reason, *p*H 7.5 was selected although the *p*H optimum of Reaction (1) was nearly 8.5.[3] Reaction (2) was optimal in the *p*H range 7.3 to 7.5. Further increase in the concentration of the reagents in the assay systems (succinate, NAD, Mg^{++} EDTA, fumarate, or NADH) did not change the rates of the two reactions.

Other properties of general interest included a slight inhibition of Reaction (1), but not of Reaction (2), by ADP or AMP (approximately 30% by 1 mM nucleotide). The inhibition of NAD reduction by succinate in the presence of ATP by relatively high concentrations of phosphate (Löw *et al.*, 1961) has been confirmed in our studies. Reaction (2), on the other hand, was unaffected by phosphate in concentrations as high as 0.05 M. Fumarate and malate did not reduce NAD when used in place of succinate in Reaction (1).

Another interesting feature of Reaction (1) is a lag before initiation of NAD reduction. Under our standard experimental conditions, which included a preincubation of ETP_H with the assay medium, the lag is 30 to 45 seconds. The first reading in the assay was taken at 2 minutes in order to avoid variability introduced by the lag. The lag varied from 1 to 6 minutes under different experimental conditions. A similar but shorter lag was observed in the reduction of pigeon heart intramitochondrial NAD by Chance and Hagihara (1960b). Hommes (1962) has reported that the lag in the ETP_H-catalyzed reaction is related to an interaction with ATP, since addition of succinate or NAD to initiate the reaction gave no lag. Also, the lag was reduced in the presence of the soluble activating factor obtained from the supernatant solution after sonic disruption of mitochondria. We have observed no lag in the assay of the fumarate-dependent oxidation of NADH (Reaction 2).

As indicated above, the activity of ETP_H in Reaction (2) did not change by the addition of 0.1 to 2.0 mM ADP, or 1 to 50 mM phosphate or both, to the reaction medium. The particles contained approximately 8 mμmoles of inorganic phosphate per mg protein, but one washing with 0.25 M sucrose reduced the level to less than 1 mμmole per mg. Even these washed particles with low endogenous phosphate showed no stimulation in activity by the addition of phosphate. Although inorganic phosphate and ADP were not obligatory for the activity, there was a net synthesis of ATP in their presence coupled to the oxidation of NADH by fumarate (Table II). The glucose-6-phosphate production was computed in experiment 1 (Table II) from the difference between two large numbers. However, the data are probably valid, since the subsequent experiments carried out with P^{32} gave similar P/2e values. Experiment 3a shows that phosphorylation could be increased by the addition of a soluble coupling enzyme which has been found to enhance the activity of ETP_H and washed ETP_H in Reaction (1) (Sanadi *et al.*, 1962). These results constitute evidence for the reversibility of Reaction (1). Since the P/2e values in all experiments were less than 1.0, the indicated stoichiometry may be correct although it is by no means established.

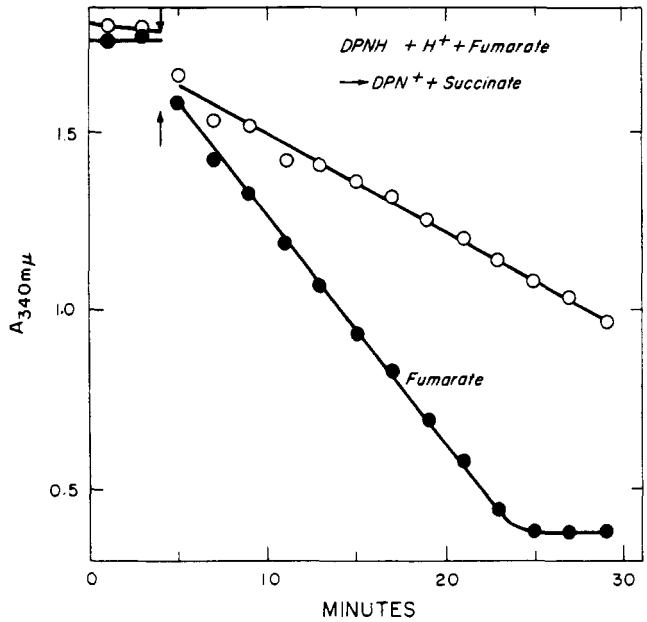

FIG. 2.—Fumarate-dependent oxidation of NADH. The assay was carried out as described in the text. The control (open circles) received 0.1 ml water instead of fumarate (solid circles).

The oxidation-reduction roles of succinate and fumarate were confirmed by use of C^{14}-labeled substrates. C^{14}-fumarate was isolated from Reaction (1) in a yield representing 70% of the NADH produced (Table III). The C^{14}-succinate obtained from Reaction (2) amounted to 86% of the NADH oxidized in the presence of fumarate.

Electron Transport Inhibitors.—Figure 3 shows that Reactions (1) and (2) are both inhibited by the usual respiratory inhibitors. Amytal and antimycin inhibit the ATP-dependent NAD reduction (Reaction 1) somewhat more strongly than they do Reaction (2). On the other hand, Reaction (2) is more sensitive to TTB. The inhibitor studies have been repeated with at least three preparations of ETP_H, and the differences in sensitivity were observed in each case. Malonate, which inhibits the succinic dehydrogenase flavoprotein (Massey and Singer, 1957), also inhibits Reactions (1) and (2) (Table IV).

The aerobic oxidations of NADH and of succinate under similar conditions (see Experimental Procedure) were far more sensitive to antimycin than were Reac-

[3] Dr. F. Hommes has also observed that the optimum *p*H for the reaction was high (private communication).

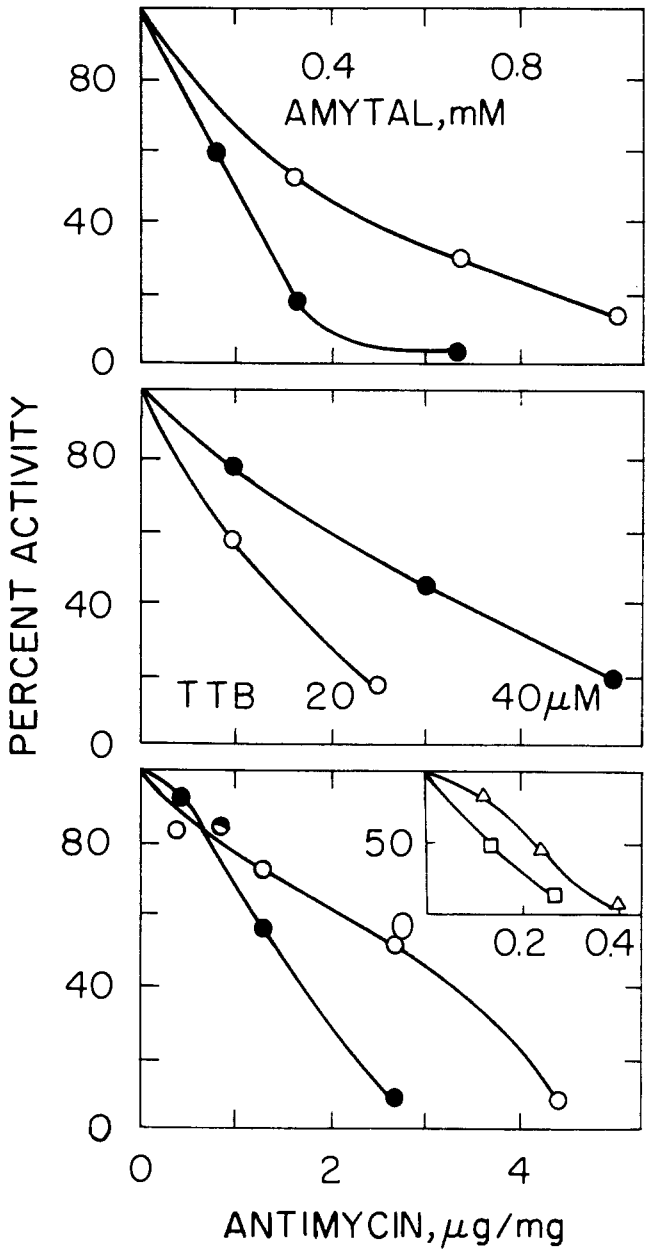

FIG. 3.—Effect of electron transport inhibitors on the reactions. The TTB and antimycin were added in 10% ethanol solution to the reaction mixture and incubated with the ETP_H as described in the text before addition of ATP or fumarate. The controls received similar levels of 10% ethanol. Solid circles represent the ATP-dependent reduction of NAD by succinate and open circles the fumarate-dependent oxidation of NADH. The triangles and squares represent the aerobic oxidation of succinate and NADH respectively (see Experimental Procedure for details).

tions (1) and (2) (Figure 3). Similarly, the reduction of cytochrome *c* by succinate was also highly sensitive. The level required for 50% inhibition was generally less than 0.25 μg antimycin per mg ETP_H protein, whereas 1.0 to 15 μg was required with four different preparations for half-maximal inhibition of Reaction (1). Similar high resistance to antimycin has been reported by Löw *et al.* (1961). Reaction (2) was also relatively insensitive to the antibiotic; the level for 50% inhibition ranged from 2.0 to 2.3 μg per mg protein with three different particles.

Uncoupling Agents.—Figure 4 shows that the uncoupling agents 2,4-dinitrophenol, dicumarol, and carbonyl cyanide *p*-chlorophenylhydrazone (Heytler and Prichard, 1962) inhibit the ATP-dependent reduction of NAD (Reaction 1) but do not affect the exergonic fumarate-dependent NADH oxidation. The effects of dinitrophenol on Reaction (1) are quite similar to those reported by Löw *et al.* (1961).

Coupling Inhibitor.—Lardy *et al.* (1958) showed that oligomycin inhibited a coupling reaction close to the final production of ATP in oxidative phosphorylation. Table IV shows that Reaction (1), which requires

TABLE II

PHOSPHORYLATION COUPLED TO THE FUMARATE-DEPENDENT OXIDATION OF NADH

Experiment 1.—The assay for NADH oxidation was carried out as described under Experimental Procedure except that the medium contained also 2 mM phosphate, 1.7 mM ADP, 10 mM glucose, and 0.5 mg hexokinase. The reaction was followed for 9 minutes and then stopped by heating in a boiling water bath for 10 minutes. The precipitated protein was removed by centrifugation, and the glucose-6-phosphate was determined in the supernatant by the glucose-6-phosphate dehydrogenase system (Kornberg, 1955). The control experiment without fumarate was carried out similarly and yielded 0.810 μmoles of glucose-6-phosphate, presumably mostly from myokinase activity. This value was subtracted from the total glucose-6-phosphate (0.868 μmoles) produced in the complete system to give the figure in the table. *Experiment 2.*—The conditions were similar to Experiment 1 except that 0.01 M phosphate containing 3.3×10^5 cpm per μmole phosphate was used. Esterified P^{32}-phosphate was determined on 0.5-ml aliquots of the supernatant solution as described by Hagihara and Lardy (1960). The organic phosphate found was 0.024 μmole in the control without fumarate, and 0.090 in the complete system. *Experiment 3.*—This was similar to Experiment 2 but incubation was longer (15 minutes). *Experiment 3a.*—The incubation medium contained 0.2 mg supernatant protein from the 100,000 × *g* centrifugation of the disrupted mitochondria. Otherwise it was identical with Experiment 3 and was carried out at the same time. The controls with no fumarate gave 0.099 μmoles organic phosphate radioactivity in both Experiments 3 and 3a.

Experiment	Δ NADH	Glucose-6-phosphate	Δ P^{32}	P/2e
	μmoles			
1	0.109	0.058		0.53
2	0.152		0.066	0.43
3	0.186		0.031	0.17
3a	0.122		0.075	0.61

TABLE III

IDENTIFICATION OF PRODUCTS

The reactions were carried out as described in the text (Experimental Procedure). The radioactivity of succinate was 1.1×10^4 cpm/μmole and of fumarate 3.7×10^4 cpm/μmole. In Reaction (1) above, the radioactivity recovered in the "fumarate" fraction from a control with no added ATP has been subtracted. The actual radioactivity recovered in the complete system and minus ATP control were 8410 and 6500 cpm respectively. Two controls were run for Reaction (2), one with no NADH and one at time 0 with no incubation. The radioactivity recovered in the two cases was 7140 and 7600 cpm respectively. In the complete system (incubated for 15 minutes) 16,550 cpm was recovered in the sublimed succinic acid.

	Δ NADH	Fumarate	Succinate
	μmoles		
Reaction (1)	0.24	0.17	
Reaction (2)			
Complete	0.28		0.24
No fumarate	0.10		

TABLE IV

INHIBITION BY MALONATE AND OLIGOMYCIN

The reactions were measured as described under Experimental Procedure.

Compound	Conc.	ΔA_{340} Reaction (1)	mμ/8 min. Reaction (2)
Malonate	—	0.224	0.126
	1.7 mM	0.006	0.019
Oligomycin	—	0.379	0.234
	0.5 μg/ml	0.000	0.211
	1.0 μg/ml	0.000	0.207

ATP, is inhibited strongly, whereas Reaction (2) is insensitive to similar levels of oligomycin (see also Löw *et al.*, 1961).

DISCUSSION

Löw *et al.* (1961) demonstrated an increase in absorbancy at 340 mμ when beef heart mitochondrial particles were incubated anaerobically with ATP, NAD, and succinate. The increase was ascribed to reduction of NAD by the succinate. Gregg and Lehninger (quoted by Lehninger and Wadkins, 1962) observed a similar change with sonic particles from rat liver mitochondria, but apparently this was caused by pronounced light-scattering changes. Figure 1 shows that the absorbancy change in a system similar to that of Löw *et al.* (1961) is indeed due to NADH formation, since addition of crystalline alcohol dehydrogenase and acetaldehyde resulted in immediate decrease of the absorbancy to almost the initial readings. If there is a light-scattering change in our system, it is probably quite small compared to the rate of NAD reduction. The complete dependence of the absorbancy change on ATP, and its prevention by oligomycin, Amytal, and agents which uncouple oxidative phosphorylation, clearly confirm that NAD reduction does proceed by reversal of oxidative phosphorylation or reductive dephosphorylation of ATP. If the reactions are analogous to those in the bacterial system of Pinchot (1960), it would appear that NAD is converted by ATP to a "high-energy" derivative which then accepts the electrons derived from succinate. Equations (3) and (4), representing the intermediate reactions, undoubtedly involve several steps. If extra energy from ATP were not available, the reduction would be thermodynamically impossible.

$$\text{ATP} + \text{NAD}^+ + \text{enzyme} \rightleftharpoons \text{ADP} + \text{P}_i + \text{enzyme} \sim \text{NAD}^+ \quad (3)$$

$$\text{enzyme} \sim \text{NAD}^+ + \text{succinate} \rightleftharpoons \text{enzyme} + \text{NADH} + \text{H}^+ + \text{fumarate} \quad (4)$$

The exergonic oxidation of NADH by fumarate is not affected by uncoupling agents and is independent of added Mg^{++}, phosphate, and ADP. However, if these compounds are presented to the system, a net esterification of phosphate is observed (Table III). Thus, a "loosely coupled" phosphorylation is associated with the electron transport between NADH and fumarate in these particles. Oxidative phosphorylation in the same over-all reaction has been observed also in extracts of *Ascaris lumbricoides* muscle (Seidman and Entner, 1961).[4]

The pathway of electron transport in Reactions (1) and (2) would appear to be the same, since both are inhibited to roughly the same extent by malonate (Table IV), Amytal, TTB, and antimycin. The observed slight differences in sensitivity could be a reflection of differences in the ratios of oxidized to reduced electron carrier when the electron flow is in opposite directions. These inhibition data together with the demonstration of ATP synthesis in the oxidation of NADH by fumarate would argue against the occurrence of a by-pass around the phosphorylating site in Reaction (2).

The inhibition of Reactions (1) and (2) by malonate certainly establishes the participation of succinic dehydrogenase flavoprotein in the electron transfer (Massey and Singer, 1957). Similarly, the NADH dehydrogenase complex is strongly implicated, since Amytal and TTB have been shown to inhibit the reduction of coenzyme Q_1(CoQ) by NADH (Hatefi *et al.*, 1962).

The significance of the inhibition of Reactions (1) and (2) by relatively *high* levels of antimycin is difficult

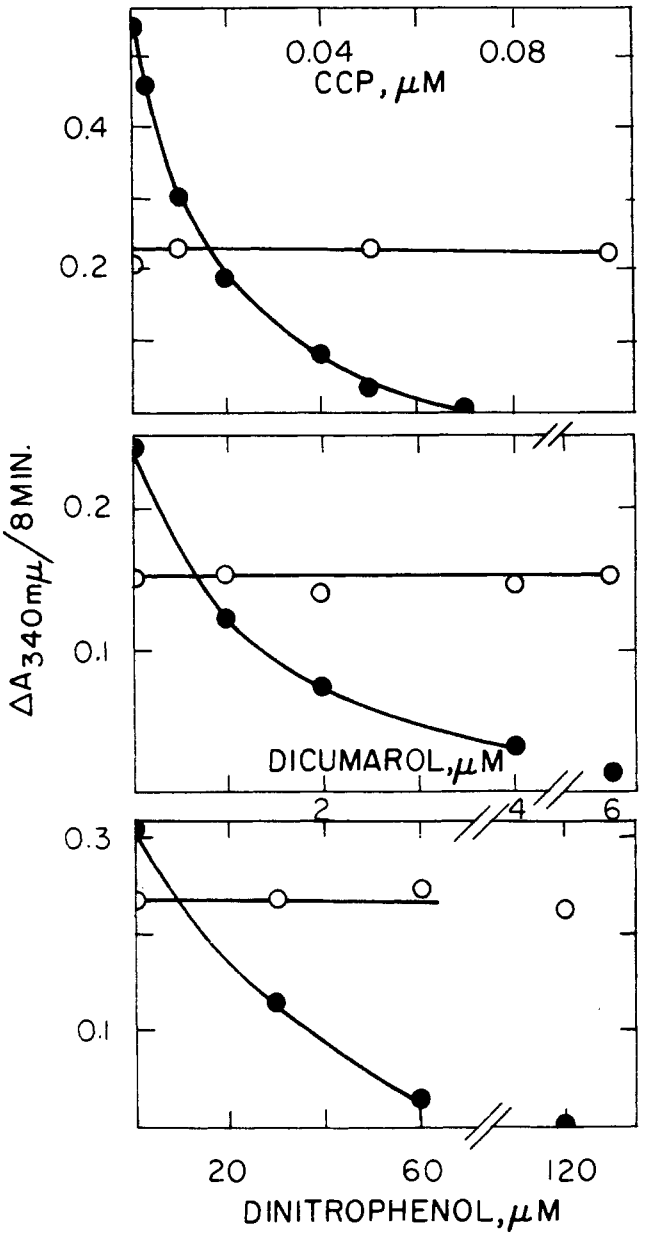

FIG. 4.—Effect of uncoupling agents. The reagents were incubated with the reaction mixture and ETP_H before the addition of ATP or fumarate as described under Experimental Procedure. Solid circles represent the ATP-dependent reduction of NAD by succinate and open circles the fumarate-dependent oxidation of NADH.

[4] Private communication from Dr. E. Bueding.

to assess, since normal respiration is highly sensitive to the antibiotic. There is a 5- to 100-fold difference in the level of antimycin that produces 50% inhibition of the aerobic oxidation by these particles as compared to the level required for a similar effect on Reactions (1) and (2). Löw and co-workers (1961) attribute the inhibition of Reaction (1) by high levels of antimycin to its uncoupling effect. Since the oxidation of NADH by fumarate (Reaction 2), which can be independent of phosphorylation, is also affected in a similar manner (Fig. 4), such an explanation does not seem completely satisfactory. Hommes (1962), on the other hand, ascribes the lower sensitivity of Reaction (1) to antimycin (compared to respiration) to a possible change in the rate-limiting step. The concept that antimycin practically binds the respiratory chain in a one-to-one relationship (Potter and Reif, 1952) would need modification if the explanation offered by Hommes is correct. Figure 4 shows that 0.4 μg of the inhibitor per mg protein, which gave nearly 100% inhibition of respiration, produced less than 15% inhibition in Reactions (1) and (2) in the identical ETP_H preparation. In this connection, it is of interest to note that the fumarate-dependent oxidation of NADH observed in extracts of *Ascaris* (Kmetec and Bueding, 1961) and *Mycobacterium avium* (Kusunose and Kusunose, 1959) is reported to be insensitive to antimycin. The mode of action of antimycin on normal respiration is supposed to be the prevention of the oxidation of reduced cytochrome *b* by c_1 (Chance and Williams, 1956). The involvement of cytochrome *b* in Reactions (1) and (2) is not inconsistent with our data if it is assumed that the reduced cytochrome *b* is oxidized in these reactions not by cytochrome c_1 but by some other acceptor. This could confer decreased sensitivity to antimycin. Alternatively, it is possible that high levels of antimycin may affect a second site between NADH and cytochrome *b* in the respiratory chain.[5] The sensitivity to higher levels of antimycin would be masked in normal respiration by the primary inhibition observed at much lower levels. In any case, the complete significance of the relatively low sensitivity to the antibiotic and the possible involvement of cytochrome *b* can be known with certainty only when the system is purified further.

Acknowledgment

The authors acknowledge the competent technical assistance of Mr. Albert Opher and Mr. Daniel Howard in these studies.

[5] Löw and Vallin (1962) recently found that reduction of NAD by ascorbate in the presence of tetramethyl-*p*-phenylenediamine, which introduces electrons in the respiratory chain at the level of cytochrome *c*, was inhibited completely by 0.17 μg antimycin/mg protein. When succinate was the electron donor, inhibition was only 45% with 4.4 μg antimycin/mg protein. The NADH-coenzyme Q_6 reductase activity of both ETP_H and digitonin extracts prepared from it is inhibited 50% by approximately 20 μg antimycin/mg protein (unpublished data). Since Hatefi *et al.* (1962) found negligible amounts of cytochrome *b* in their purified NADH-CoQ reductase, the possibility of the existence of a second site for antimycin action in the electron transport chain indeed needs serious consideration.

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