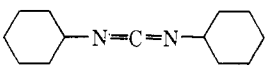
The Properties of Dicyclohexylcarbodiimide as an Inhibitor of Oxidative Phosphorylation*

R. B. Beechey, A. M. Roberton, Caroline T. Holloway,† and I. G. Knight

ABSTRACT: Dicyclohexylcarbodiimide (DCCD) is an inhibitor of oxidative phosphorylation. At concentrations around 2 mμmoles/mg of protein it inhibits the coupled respiration of intact mitochondria. Uncoupling agents relieve this inhibition of electron transport but arsenate has no effect. DCCD does not affect the substrate-linked phosphorylation associated with the oxidation of α-oxoglutarate. Three or four molecules of DCCD per electron transport chain are sufficient to inhibit the adenosine triphosphate (ATP) driven partial reactions of oxidative phosphorylation, the adenosine triphosphatase, and P_i–ATP-exchange reactions in submitochondrial particles. At these concentrations DCCD stimulates the aerobic energy-linked pyridine nucleotide transhydrogenase activity. The onset of inhibition varies with time, temperature, and pH of incubation of mitochondria or particles with DCCD. DCCD (0.2 mμmole/mg of protein) is enough to stimulate the ATP-driven partial reactions. DCCD is thought to react with an intermediate of oxidative phosphorylation at the level of a phosphorylated intermediate forming covalent bonds.

Dicyclohexylcarbodiimide (DCCD)[1] (I) has been shown to inhibit the ADP-stimulated respiration of tightly coupled mitochondria when oxidizing the following substrates: succinate in the presence of rotenone, glutamate plus malate in the presence of



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malonate, and ascorbate in the presence of TMPD. DCCD also inhibits the ATP-driven partial reactions of oxidative phosphorylation catalyzed by submitochondrial particles. Thus DCCD acts in a manner very similar to oligomycin. All these inhibitions are immediate at concentrations around 40 mμmoles of DCCD/mg of protein (Beechey *et al.*, 1966). One site of action of DCCD has been localized in the CF_0 complex first isolated by Kagawa and Racker (1966; Roberton *et al.*, 1966). Other experiments have shown that in contrast to oligomycin (Kagawa and Racker, 1966), the inhibitory effects of DCCD are not reversible and that [^{14}C]DCCD binds to the mitochondria (Holloway *et al.*, 1966). Thus DCCD shows some promise as a potential label of one of the intermediates in oxidative phosphorylation.

In this paper we present evidence which shows that low concentrations of DCCD (approximately 1 mμmole of DCCD/mg of protein) will inhibit both the synthesis of ATP in mitochondria and the ATP-driven partial reactions of oxidative phosphorylation catalyzed by submitochondrial particles, after preincubation of the mitochondrial preparation with the inhibitor. DCCD does not affect the substrate-linked synthesis of ATP associated with the oxidation of α-oxoglutarate. DCCD exerts its effects on oxidative phosphorylation by the carbodiimide moiety of the molecule reacting at the level of a phosphorylated intermediate. Some of these results have been reported briefly elsewhere (Roberton *et al.*, 1966).

Methods

Preparation of Heart Mitochondria, Submitochondrial Particles, and EDTA Particles. Mitochondria were prepared from ox heart either by the method of Sanadi and Fluharty (1963) or by the Nagarse proteinase procedure described by Beinert in Umbreit *et al.* (1964). The mitochondria were separated into light and heavy layer fractions. Rat heart mitochondria were prepared by the method of Chance and Hagihara (1961). Submitochondrial particles were prepared by disintegration of heavy layer mitochondria by sonic oscillation, essentially as described by Hansen and Smith (1964). Unless otherwise stated the submitochondrial particles were suspended in a medium containing 250 mM sucrose, 1 mM K-ATP, 1 mM potassium succinate, 5 mM magnesium chloride, and 10 mM Tris-chloride (pH 7.6). EDTA particles were prepared by the method of Lee *et al.*, (1964) from

* From Shell Research Limited, Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent, England. *Received July 17, 1967.*

† Milstead Research Fellow.

[1] Abbreviations: DCCD, dicyclohexylcarbodiimide; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; As_i, arsenate; ADP and ATP, adenosine di- and triphosphates; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotides; $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphates.

heavy layer mitochondria which had been prepared the previous day by the method of Sanadi and Fluharty (1963) and stored overnight at 0°. The particles were suspended in a solution containing 250 mM sucrose and 10 mM Tris-sulfate (pH 7.7).

Assay of Protein. Protein was estimated colorimetrically by the biuret method of Gornall *et al.* (1949), after solubilization of the protein with 1.5% (w/v) potassium cholate.

Assay of Respiration Rates. A Clark oxygen electrode was used to measure oxygen concentrations in an apparatus similar to that described by Chappell (1964). The temperature of the reaction medium was 25°. Additions to the reaction vessel were made with microsyringes. The pH of all the aqueous solutions was adjusted to 7.4 with potassium hydroxide. Following the method of Chappell (1964) the oxygen concentration of the air-saturated reaction media was measured and found to be 237 μM at 25°, a result identical with that reported by Chappell.

Assay of Adenosine Triphosphatase Activity. Two methods were used. In the first, 3.2 μmoles of ATP was added to the reaction medium and hence the concentration of ATP decreased during the reaction period. In the second an ATP-regenerating system maintained the ATP concentration at a constant level (Pullman *et al.*, 1960). (1) Submitochondrial particles (0.1–0.2 mg of protein), suspended in 0.2 ml of a solution containing 250 mM sucrose, 1 mM K-ATP, 1 mM potassium succinate, 5 mM magnesium chloride, and 10 mM Tris-chloride (pH 7.4), were added to 0.8 ml of a reaction mixture containing 200 mM sucrose, 100 mM Tris-chloride (pH 7.4), and 4 mM ATP. The temperature was 30° and the reaction time 15 min. The reaction was stopped by the addition of 0.1 ml of 40% (w/v) trichloroacetic acid. After centrifuging, the P_i content of the supernatant was assayed by the method of Sumner (1944). (2) A reaction mixture (0.95 ml) containing 50 mM Tris-chloride (pH 7.4), 6 mM K-ATP, 3 mM magnesium chloride, 5 mM potassium phosphoenolpyruvate, and 100 μg of pyruvate kinase was equilibrated at 30° for 5 min, then 0.05 ml of a suspension of submitochondrial particles (0.85–1 mg of protein) was added. After 15 min, the reaction was stopped by the addition of 0.1 ml of 40% (w/v) trichloroacetic acid. After centrifuging, the P_i content of the supernatant was assayed.

Assay of the Nonenergy-Linked Pyridine Nucleotide Transhydrogenase. This and the following procedures are based on the methods described by Danielson and Ernster (1963). The reaction mixture contained 250 mM sucrose, 5 mM magnesium chloride, 50 mM Tris-chloride or Tris-sulfate buffer (pH 8.0), 0.66 μM rotenone (recrystallized twice from ethanol), 400 μM redistilled ethanol, 66 μM NAD^+, 300 μg of yeast alcohol dehydrogenase, and 1 mM potassium cyanide; temperature 30°. Submitochondrial particles (0.5–1 mg of protein) were added, followed after 1 min by $NADP^+$ to a final concentration of 330 μM. The final volume was 3 ml. Reduction of $NADP^+$ was measured by the change in extinction at 340 mμ using a Zeiss PMQ II or a Unicam S.P. 800 spectrophotometer.

Assay of the ATP-Driven Pyridine Nucleotide Transhydrogenase. The assay was the same as that used in the nonenergy-linked pyridine nucleotide transhydrogenase assay, except that ATP (final concentration 1.33 mM) was added 1 min after the $NADP^+$. The rates quoted here have been corrected for rates of the nonenergy-linked pyridine nucleotide transhydrogenase. This correction was never greater than 15% of the ATP-driven pyridine nucleotide transhydrogenase.

Assay of the Aerobic Energy-Linked Pyridine Nucleotide Transhydrogenase. The assay was the same as for the nonenergy-linked pyridine nucleotide transhydrogenase, except that cyanide was omitted and that potassium succinate (final concentration 1.33 mM) was added before the submitochondrial particles.

Assay of the ATP-Driven Reduction of NAD^+ by Succinate. This method is based on that described by Löw and Vallin (1963). A reaction mixture containing 250 mM sucrose, 6 mM magnesium chloride, 50 mM Tris-chloride (pH 8.0), 1 mM potassium cyanide, 1 mM NAD^+, and 5 mM potassium succinate was equilibrated at 30° for 3 min. Either submitochondrial particles or EDTA particles (0.5–1 mg of protein) were added, followed by ATP to a final concentration of 2 mM. The final volume was 3 ml. The change in extinction at 340 mμ was measured.

Assay of the ATP-Driven Reduction of NAD^+ by TMPD Plus Ascorbate. The method of Löw *et al.* (1963) was used.

Assay of P_i–ATP-Exchange Activity. Carrier-free $[^{32}P]P_i$ was purified by the method of Conover *et al.* (1963). The following procedure for measuring $[^{32}P]P_i$–ATP exchange is based on the method of Mr. J. Haslam (personal communication). A reaction mixture containing 250 mM sucrose, approximately 5 mM $[^{32}P]P_i$ ($2–4 \times 10^5$ dpm/μmole), 1 mM ATP, 1.2 mM ADP, 4 mM magnesium sulfate, and 50 mM Tris-sulfate (pH 7.6) was equilibrated at 30°. A 0.1-ml sample of submitochondrial particles (0.1–0.2 mg of protein) was added to 0.9 ml of the reaction mixture. After 10 min the reaction was stopped by addition of 2 ml of a solution containing 0.9 M perchloric acid and 0.6 M sodium sulfate. In the control incubation, the submitochondrial particles were added after addition of acid at 10 min. The acidified mixtures were cooled to 0° and the protein was removed by centrifuging. $[^{32}P]P_i$ incorporation into adenine nucleotide was estimated as described below.

The Separation of $[^{32}P]$Adenine Nucleotides from $[^{32}P]P_i$. The phosphomolybdate complexes of the adenine nucleotides and P_i were formed and then separated by solvent extraction. An ammonium molybdate solution (1.5 ml) (reagent B of Hagihara and Lardy, 1960) was added to 1.5 ml of the acidified supernatant solution from the reaction assay. The mixture was allowed to stand at 4° for 10 min away from strong light. This solution was then extracted in the cold room five times with the phosphomolybdic acid extraction reagent described by Hagihara and Lardy (1960) using 1-hexanol as the organic solvent.

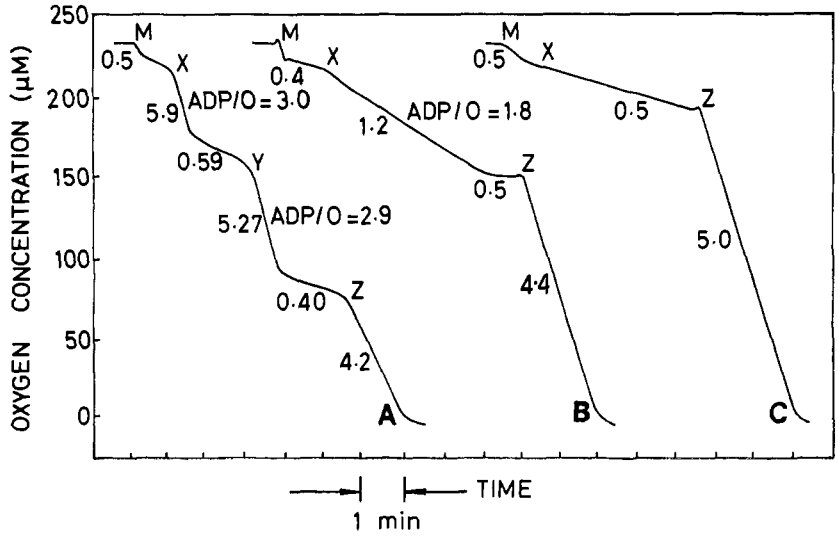


FIGURE 1: Inhibition by DCCD of the stimulation of mitochondrial respiration by ADP. Heavy beef heart mitochondria were prepared by the proteinase method and suspended in 250 mM sucrose (500 mg of protein/6.6 ml). To three 6.6-ml aliquots of this mitochondrial suspension was added either 10 μl of dimethylformamide or 5 or 10 μl of a 0.1 M DCCD solution in dimethylformamide. After mixing, the mitochondrial suspensions were stored at 0° for 24 hr. The respiration rates of the mitochondrial suspensions at 25° were then measured using an oxygen electrode; the traces above represent the output from the electrode. The mitochondrial suspensions (50 μl) were added to an aerated reaction mixture which contained 250 mM sucrose, 7.5 mM potassium phosphate (pH 7.4), 10 mM magnesium chloride, 2.5 mM potassium L-glutamate, 2.5 mM potassium L-malate, and 5 mM potassium malonate. The final volume was 4.16 ml. The numbers on the curves represent the respiration rates expressed as micromoles of oxygen per milligram of protein per hour at 25°. The following additions were made as indicated: at M, mitochondria; at X, 25 μl of 40 mM ADP; at Y, 50 μl of 40 mM ADP; and at Z, 10 μl of 10 mM 2,4,5-tribromoimidazole in ethanol. Trace A, control mitochondria; trace B, mitochondria treated with 1 mμmole of DCCD/mg of protein; trace C, mitochondria treated with 2 mμmoles of DCCD/mg of protein.

Before the third and fourth extractions 4 μmoles of carrier P_i was added to the aqueous phase. This aided the P_i extraction by dissociating ternary adenine nucleotide–phosphomolybdate complexes. An aliquot of the final aqueous phase was counted.

The final ATP content of the acidified supernatant was assayed enzymically and the results were calculated as micromoles of P_i incorporated into adenine nucleotide per micromole of ATP measured at the end of the reaction.

Measurement of P:O Ratios. This was based on the method described by Pullman and Racker (1956). Oxygen consumption was measured with an oxygen electrode inserted into a Perspex cubiodal cell 2.92 cm high × 1.36 cm wide × 1.89 cm light path. This cell was inserted into the cuvet holder of a Zeiss PMQ II spectrophotometer which had been fitted with a magnetic stirrer to stir the contents of the cell. ATP synthesis was measured by following the changes in extinction at 340 mμ caused by the production of NADPH thus

$$\text{ATP} + \text{glucose} \longrightarrow \text{glucose 6-phosphate} + \text{ADP}$$
$$\text{glucose 6-phosphate} + \text{NADP}^+ \longrightarrow$$
$$\text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

A stock reagent solution was prepared containing the following amounts in 1.6 ml: 50 μmoles of magnesium sulfate, 100 μmoles of sodium phosphate (pH 7.4), 10 mg of crystalline bovine plasma albumin, 2 mg of crystalline hexokinase (Sigma type III), 200 μmoles of glucose, 2 μmoles of $NADP^+$, 50 μmoles of AMP, and 125 μmoles of sucrose.

The following were added to the spectrophotometer oxygen electrode cell: 1.6 ml of stock reagent solution, 5.7 ml of 250 mM sucrose, 0.1 ml of submitochondrial particles (2–3 mg of protein), and 5 μl of glucose 6-phosphate dehydrogenase (5 μg of protein). After 0.5 min, 10 μl of 40 mM ADP was added. Any small changes in extinction at this point are due to the adenylate kinase activity of the submitochondrial particles; however, the presence of AMP in the reaction medium rendered these changes very small. Substrate was then added, either 0.1 ml of 0.1 M potassium succinate or 1 μl of 20 mM NAD^+ plus 20 μl of ethanol and 5 μl of yeast alcohol dehydrogenase (150 μg of protein). P:O ratios were calculated from the ratio of the rate of NADPH formation to the rate of oxygen consumption, when both rates were linear.

Assay of ADP:O Ratios. The method of Chance and Williams (1955) was used.

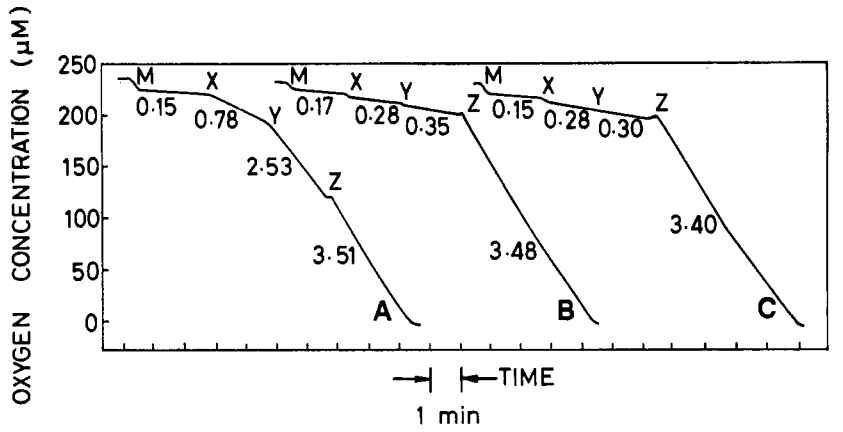


FIGURE 2: Inhibition by DCCD of the stimulation of mitochondrial respiration by arsenate. Heavy beef heart mitochondria were prepared by the proteinase method and suspended in 250 mM sucrose containing 10 mM Tris-sulfate (pH 7.6) (59.2 mg of protein/ml). To three 0.5-ml aliquots of this suspension were added 0, 1, and 2 mμmoles of DCCD/mg of protein dissolved in 3.0 μl of ethanol. The mitochondria were stored at 0° for 24 hr. The respiration rates of the three preparations were then measured using an oxygen electrode; the traces above represent the output from the electrode, and the numbers represent respiration rates expressed as micromoles of oxygen per milligram of protein per hour at 25°. The mitochondrial suspensions (50 μl) were added to an aerated reaction mixture which contained 250 mM sucrose, 10 mM Tris-sulfate (pH 7.6), 2.5 mM potassium L-glutamate, 2.5 mM potassium L-malate, and 5 mM potassium malonate. The final volume was 3.6 ml. The following additions were made as indicated: at M, mitochondria; at X, 40 μl of 0.1 M sodium arsenate (pH 8); at Y, 10 μl of 40 mM ADP; and at Z, 10 μl of 10 μM TTFB in ethanol. Trace A, control mitochondria; trace B, mitochondria treated with 1 mμmole of DCCD/mg of protein; trace C, mitochondria treated with 2 mμmoles of DCCD/mg of protein.

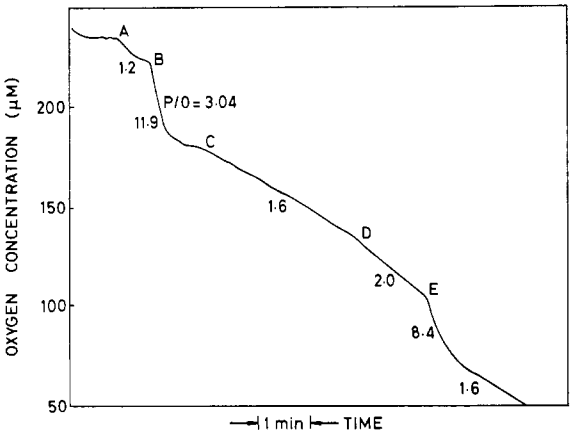


FIGURE 3: Substrate-linked phosphorylation in DCCD-treated rat heart mitochondria. The trace represents the output from an oxygen electrode. At A, rat heart mitochondria (2.27 mg of protein) suspended in 75 μl of 250 mM sucrose containing 1 mM EDTA were added to 4 ml of an aerated reaction mixture containing 240 mM sucrose, 10 mM potassium phosphate (pH 7.4), and 10 mM α-oxoglutarate. At B, 25 μl of 40 mM K-ADP (1 μmole) was added. At C, 4 μl of a 0.1 M DCCD solution in dimethylformamide was added (176 mμmoles of DCCD/mg of protein). At D, 15 μl of 10 mM 2,4-dinitrophenol was added. At E, 5 μl of 40 mM K-ADP (0.2 μmole) was added. The numbers represent the respiration rate expressed in micromoles of oxygen per milligram of protein per hour at 25°.

Materials

DCCD was purchased from a number of British, European, and U. S. chemical suppliers. All the samples had similar activity in the enzyme systems studied. Routinely DCCD was dissolved in either dimethylformamide or ethanol to give a concentration of 10 mM. Aliquots of this solution were added to suspensions of mitochondria and submitochondrial particles to give the required final concentrations of DCCD.

Oligomycin A was a gift of Professor E. E. van Tamelen.

Rutamycin was a gift of the Lilly Research Laboratories.

Results

Effect of DCCD on the Coupled Respiration of Mitochondria. We have previously shown (Beechey *et al.*, 1966) that DCCD will immediately inhibit ADP-stimulated mitochondrial respiration (state 3 of Chance and Williams, 1955) when added at concentrations around 40 mμmoles of DCCD/mg of protein. Subsequent experiments have shown that the concentration of DCCD needed for inhibition is lower if the mitochondria are preincubated with DCCD. This is illustrated in Figure 1. DCCD dissolved in dimethylformamide was added to suspensions of freshly prepared heavy beef heart mitochondria to give concentrations

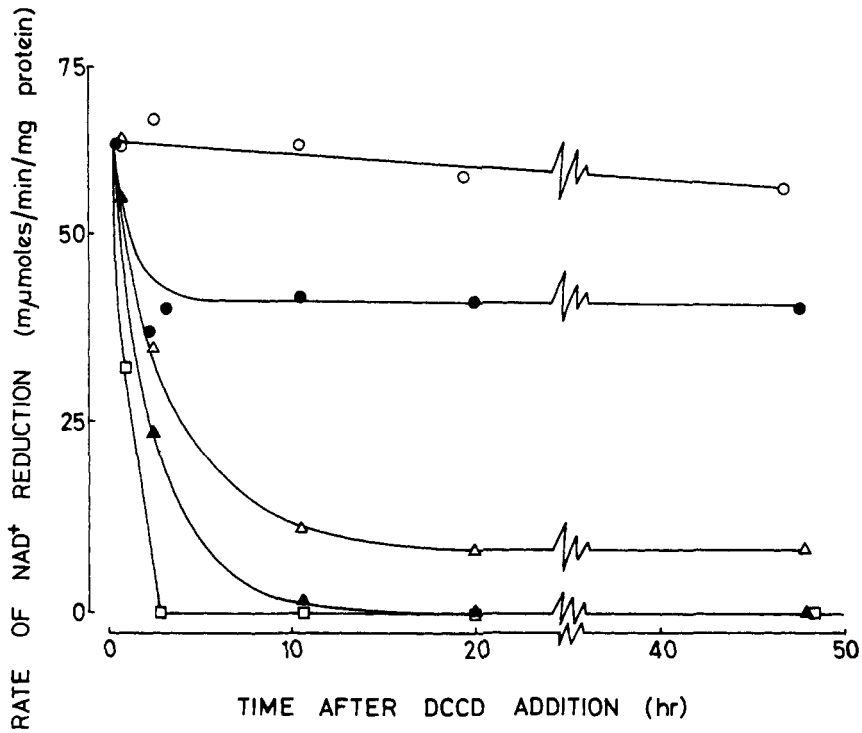


FIGURE 4: Effect of preincubation of submitochondrial particles with DCCD on the rate of ATP-driven reduction of NAD^+ by succinate. Aliquots of a 10 mM solution of DCCD in dimethylformamide were added to 1-ml samples of submitochondrial particles (3.56 mg of protein/ml) to give the following concentrations of DCCD: 2 mμmoles/mg of protein (□), 1 mμmole/mg of protein (▲), 0.66 mμmole/mg of protein (△), 0.33 mμmole/mg of protein (●), and control to which dimethylformamide was added (○). After the addition of DCCD the samples were stored at 0° and aliquots were removed for assay at the times shown.

of 1 and 2 mμmoles of DCCD/mg of protein; dimethylformamide was added to the control suspension of mitochondria. The respiration rates, the respiratory control ratios, and the ADP:O ratios of the control and DCCD-treated mitochondria were measured immediately after the addition of dimethylformamide or DCCD solution and 24 hr later. The substrate was an equimolar (2.5 mM) solution of L-glutamate and L-malate in the presence of 5 mM malonate. The addition of DCCD had no immediate effect on the respiration rates, respiratory control, and the ADP:O ratios. After incubation for 24 hr the behavior of the control mitochondria had changed little (Figure 1, curve A). However, whereas the respiration rate of the DCCD-treated mitochondria was unaffected in the absence of ADP (state 4 of Chance and Williams, 1955), the ability of ADP to stimulate respiration was inhibited. DCCD at the level of 1 mμmole/mg of protein gave a partial inhibition, with a lowering of the ADP:O ratio (Figure 1, curve B). Two millimicromoles of DCCD per milligram of protein completely inhibited the ability of ADP to stimulate respiration (Figure 1, curve C). The addition of an uncoupling concentration of 2,4,5-tribromoimidazole (Beechey, 1966) gave an immediate stimulation of the respiration rate. The prior addition of ADP was not a prerequisite for the stimulation of the inhibited respiration rate by uncoupling agents. Dicoumarol, 2,4-dinitrophenol, and TTFB also relieved the DCCD inhibition of mitochondrial respiration. Similar results have been obtained on the inhibition of respiration by DCCD with succinate as the substrate. The inhibition of ADP-stimulated respiration was not reversed by bovine plasma albumin.

Inhibition by DCCD of Arsenate-Uncoupled Respiration. The experiment illustrated in Figure 2 shows that the respiration rate of heavy beef heart mitochondria stimulated by arsenate, in the presence of ADP and the absence of P_i, is completely inhibited in mitochondria which have been preincubated with either 1 or 2 mμmoles of DCCD/mg of protein. The addition of an uncoupling amount of TTFB immediately stimulates the respiration to a rate which is greater than in the presence of arsenate and ADP.

Substrate-Linked Phosphorylation in DCCD-Treated Mitochondria. Figure 3 illustrates the results of an experiment in which rat heart mitochondria were oxidizing α-oxoglutarate in the presence of P_i. The first addition of ADP, at B, shows that the mitochondria have good respiratory control and that they phosphorylate ADP with a high efficiency, ADP:O 3.04. A very high concentration of DCCD (176 mμmoles/mg of protein) added at C slightly stimulated the rate of respiration. The subsequent addition of an uncoupling concentration of 2,4-dinitrophenol, at D, had little

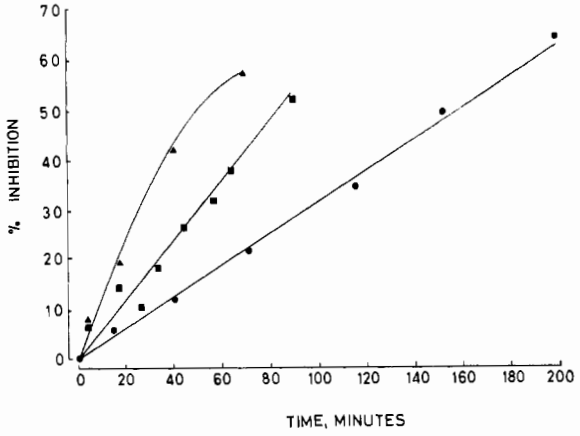


FIGURE 5: Effect of temperature on the rate of onset of DCCD inhibition of the ATP-driven reduction of NAD^+ by succinate. Aliquots of a 10 mM solution of DCCD in dimethylformamide were added to three samples of a suspension of submitochondrial particles (20 mg of protein/ml) giving a final concentration of 0.7 mμmole of DCCD/mg of protein. Dimethylformamide was added to three control samples of submitochondrial particles. The DCCD-treated and control submitochondrial particles were then kept at 0° (●), 10° (■), and 20° (▲) and aliquots were removed at the time shown for the assay of ATP-linked reduction of NAD^+ by succinate.

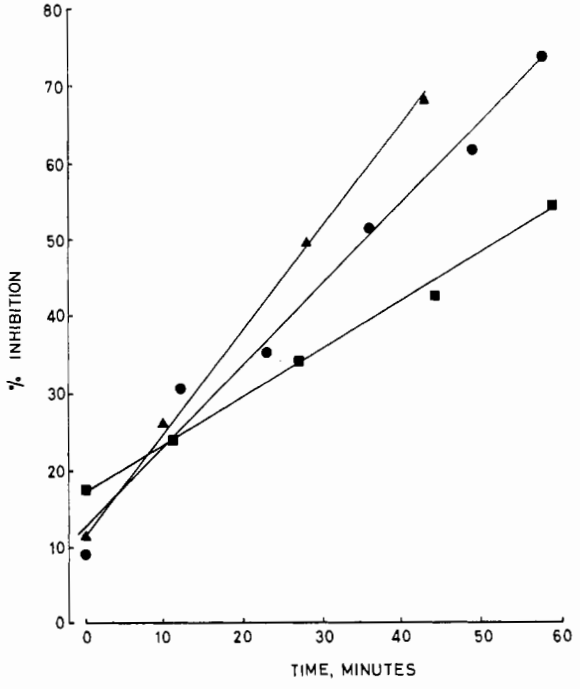


FIGURE 6: Effect of pH on the rate of onset of DCCD inhibition of the ATP-driven reduction of NAD^+ by succinate. The pH values at 0° of three samples of a suspension of submitochondrial particles were adjusted with 0.1 M Tris to 7.3, 7.88, and 8.68 (the buffer anion of the suspension medium was sulfate). The final protein concentration was 25 mg/ml. To one-half of each sample was added 0.7 mμmole of DCCD/mg of protein (using a 10 mM DCCD solution in dimethylformamide). An equal volume of dimethylformamide was added to the control samples. The submitochondrial particle samples were stored at 0° and aliquots were removed for assay at the indicated times. (▲) pH 7.30, (●) 7.88, and (■) 8.68.

effect on the respiration rate. This is to be contrasted with the effects of uncoupling agents on DCCD-inhibited respiration shown in Figures 1 and 2 where the respective substrates were glutamate plus malate, and succinate. A further addition of ADP, at E, gave a transient stimulation of the respiration rate, with an ADP:O of 0.74. In the presence of an uncoupling agent this respiratory control can only be ascribed to substrate-linked phosphorylation, associated with the oxidation of α-oxoglutarate. Thus, the latter process is not affected by DCCD.

Time Course of DCCD Inhibition. Beechey *et al.* (1966) showed that DCCD will also inhibit the ATP-driven partial reactions of oxidative phosphorylation. Because of the simplicity of measuring the DCCD inhibition of these reactions as compared with the DCCD inhibition of ADP-stimulated respiration, the time course of DCCD inhibition of the ATP-driven NAD^+ reduction by succinate reaction was examined. Figure 4 shows the results of an experiment in which submitochondrial particles were incubated at 0° for 48 hr with 0, 0.33, 0.66, 1, and 2 mμmoles of DCCD/mg of protein. The ability of the submitochondrial particles to catalyze the ATP-driven reduction of NAD^+ by succinate was measured at various times after the addition of DCCD. At all times there was a graded inhibition of this reaction; the greater the concentration of DCCD the greater the inhibition.

Effect of Temperature on DCCD Inhibition. The rate of onset of the DCCD inhibition of the partial reactions of oxidative phosphorylation was influenced by the temperature at which the submitochondrial particles were preincubated with DCCD. The results presented in Figure 5 show that the rate of inhibition by DCCD of the ATP-driven reduction of NAD^+ by succinate approximately doubles with every 10° rise in temperature. At 20° the inhibition did not increase quite linearly with time, the control preparation being slowly inactivated at this temperature.

Effect of pH on the Rate of Onset of DCCD Inhibition. We were unable to obtain quantitatively reproducible results in five experiments designed to study the effect of pH on the rate of onset of DCCD inhibition. However, the effect of pH was always qualitatively similar. Figure 6 shows the best experiment. The pH's of three samples of a suspension of submitochondrial particles were adjusted to 7.3, 7.88, and 8.68. DCCD was added to half of each sample, giving a final concentration of 0.7 mμmole/mg of protein. The three control samples of submitochondrial particles and the three samples

TABLE I: Effect of DCCD on the Synthesis of ATP, the ATP-Driven Reduction of NAD^+, and the ATP-Driven Pyridine Nucleotide Transhydrogenase Catalyzed by Submitochondrial particles.[a]

DCCD Concn (mμmoles/mg of protein)	P:O Ratio		mμmoles/min per mg of protein	
	Succinate Oxidn	NADH Oxidn	ATP-Driven Reduction of NAD^+ by Succinate	ATP-Driven Pyridine Nucleotide Transhydrogenase
0	0.19	0.50	80	130
0.5	0.17	0.40	34	82
1.0	0.09	0.20	2	19
2.0	0.02	0.03	0	0

[a] Aliquots of a 10 mM DCCD solution in dimethylformamide were added to samples of a suspension of submitochondrial particles (36.2 mg of protein/ml) to give final concentrations of 0.5, 1, and 2 mμmoles of DCCD/mg of protein. Dimethylformamide was added to the suspensions of control and the experimental submitochondrial particles to equalize the concentration of dimethylformamide at 7.25 μl/ml in all the samples of submitochondrial particles. The latter were incubated at 0° for 20 hr and then used for the enzymic assays shown.

of DCCD-treated submitochondrial particles were incubated at 0°. At intervals, aliquots were removed for the assay of the ATP-driven reduction of NAD^+ by succinate activity. The results show that the rate of onset of the inhibition of this reaction by DCCD is increased approximately twofold by decreasing the pH of incubation with DCCD from 8.68 to 7.3.

Effect of DCCD on the Synthesis of ATP and the ATP-Driven Partial Reactions of Oxidative Phosphorylation Catalyzed by Submitochondrial Particles. The data in Table I summarize the results of an experiment in which submitochondrial particles were incubated for 20 hr at 0° with 0, 0.5, 1, and 2 mμmoles of DCCD/mg of protein. The activities of the following enzyme systems were then assayed.

ATP Synthesis Linked to the Oxidation of Either Succinate or NADH. The efficiency of ATP synthesis (P:O ratio) during the oxidation of either succinate or NADH diminished almost to zero as the concentration of DCCD was increased to 2 mμmoles of DCCD/mg of protein.

ATP-Driven Reduction of NAD^+ by Succinate. This system was the most sensitive assayed, almost complete inhibition being noted after preincubation of the submitochondrial particles with 1 mμmole of DCCD/mg of protein.

ATP-Driven Pyridine Nucleotide Transhydrogenase. Complete inhibition of this reaction was noted after preincubation with 2 mμmoles of DCCD/mg of protein.

ATP-Driven Reduction of NAD^+ by TMPD Plus Ascorbate. The results presented in Table II show that the preincubation of 1 mμmole of DCCD/mg of protein for 18 hr at 0° is sufficient to inhibit completely the ATP-driven reduction of NAD^+ by TMPD plus ascorbate.

Effect of DCCD on the Aerobic Energy-Linked Pyridine Nucleotide Transhydrogenase. After preincubation of submitochondrial particles and EDTA particles with 0.5–2 mμmoles of DCCD/mg of protein at 0° for 24 hr, the activity of the aerobic energy-linked pyridine nucleotide transhydrogenase driven by succinate oxidation was invariably higher in DCCD-treated particles than in the untreated particles (Table III). The extent of activation varied considerably with different preparations of particles, the range of activation varying from 10 to 150%.

Effect of DCCD on the Nonenergy-Linked Pyridine Nucleotide Transhydrogenase. It can be seen from the

TABLE II: Effect of DCCD on the ATP-Driven Reduction of NAD^+ by TMPD Plus Ascorbate.[a]

DCCD Concn (mμmoles/mg of protein)	ATP-Driven Reduction of NAD^+ by TMPD + Ascorbate (mμmoles/min per mg of protein)
0	65
0.2	45
0.5	25
0.7	11
1.0	0

[a] Aliquots of 10 mM DCCD dissolved in ethanol were added to 1-ml samples of submitochondrial particles (16.6 mg of protein/ml) to give final concentrations of 0.2, 0.5, 0.7, and 1 mμmole of DCCD/mg of protein. Ethanol was added to the suspensions of control and the experimental submitochondrial particles to equalize the concentration of ethanol at 5 μl/ml in all samples. The submitochondrial particles were incubated for 18 hr at 0° before assaying for ATP-driven reduction of NAD^+ by TMPD plus ascorbate.

TABLE III: Effect of DCCD on the Aerobic Energy-Linked Transhydrogenase and the Nonenergy-Linked Transhydrogenase Activities of Submitochondrial Particles and EDTA Particles.[a]

Type of Particles	DCCD concn (mμmoles/mg of protein)	mμmoles/min per mg of protein: Aerobic Energy-Linked Pyridine Nucleotide Transhydrogenase	mμmoles/min per mg of protein: Nonenergy-Linked Pyridine Nucleotide Transhydrogenase
SMP	0	92	18
	0.5	99	21
	2.0	100	18
	0	86.5	10
	1.0	181	10
EDTA particles	0	69	9
	0.5	91	11
	1.0	108	11
	2.0	119	10

[a] Various preparations of submitochondrial particles (SMP) and EDTA particles were preincubated with DCCD for 24 hr at 0°, as described in the legend to Table I, before assaying.

data presented in Table III that the treatment with DCCD of either submitochondrial particles or EDTA particles has no effect on the nonenergy-linked transhydrogenase activity.

Effect of DCCD on the Adenosine Triphosphatase Activity of Submitochondrial Particles. The adenosine triphosphatase activity of submitochondrial particles is inhibited by preincubation of the particles with DCCD. The data in Table IV show that the extent of the inhibition increases progressively with increasing DCCD concentration and that the enhanced adenosine triphosphatase activity caused by uncoupling agents, such as 2,4-dinitrophenol, is equally sensitive to DCCD.

TABLE IV: Effect of DCCD on the Adenosine Triphosphatase Activity of Submitochondrial Particles.[a]

mμmoles of DCCD/mg of protein	ATPase Act. (mμmoles of P_i/min per mg of protein) Expt I −DNP	Expt I +DNP	Expt II −DNP
0	88	120	108
0.2	93	95	
0.4	57	21	
1.0	28	22	
2.0			7.2

[a] Submitochondrial particles suspended in 250 mM sucrose and 10 mM Tris-chloride (pH 7.6) were preincubated at 0° for 24 hr with the indicated concentrations of DCCD. In expt I, the adenosine triphosphatase activity was assayed with a decreasing ATP level (method 1) in the presence and absence of 100 μM 2,4-dinitrophenol (DNP). In expt II the adenosine triphosphatase activity was measured using an ATP-regenerating system (method 2). In expt I and II, 0.17 and 0.85 mg of protein of submitochondrial particles were used, respectively.

Effect of DCCD on P_i–ATP-Exchange Activity of Submitochondrial Particles. The exchange of P_i with the phosphate in the γ position of ATP, under conditions in which no net phosphorylation can occur, is thought to be catalyzed by the two terminal reactions of the oxidative phosphorylation sequence (Wadkins and Lehninger, 1958). Table V lists the results of an experiment in which submitochondrial particles were preincubated with 2 mμmoles of DCCD/mg of protein. The P_i–ATP-exchange activity was completely abolished. The control assays in which either antimycin A or cyanide was added show that the [^{32}P]P_i incorporation measured was not due to net ATP synthesis coupled to respiration.

Stoichiometry of the Reaction of DCCD with Submitochondrial Particles. Various concentrations of DCCD (0–1 mμmole/mg of protein) were added to aliquots of a suspension of submitochondrial particles and incubated for 18 hr at 0°. The activities of the following partial reactions of oxidative phosphorylation were then assayed: the ATP-driven reduction of NAD^+ by succinate, the ATP-driven reduction of NAD^+ by TMPD plus ascorbate, and the ATP-driven pyridine nucleotide transhydrogenase. The results are illustrated in Figure 7. It can be seen that 0.8–0.9 mμmole of DCCD/mg of protein is sufficient to inhibit the ATP-driven reduction of NAD^+ either by succinate or TMPD plus ascorbate. However a higher concentration

TABLE V: Effect of DCCD on the P_i–ATP-Exchange Activity of Submitochondrial Particles.[a]

	P_i–ATP-Exchange Act. (mμmoles/min per mg of protein)			
	Control SMP		DCCD-Treated SMP	
mg of SMP Protein Added	0.1	0.2	0.1	0.2
Additions				
None	68.7	68.7	0	0
2 μg of antimycin A	77.3	60.3		
1 mM KCN	55.9	57.0		

[a] A 10 mM DCCD solution (8 μl) in dimethylformamide was added to 1 ml of a suspension of submitochondrial particles (40 mg of protein) to give a concentration of 2 mμmoles of DCCD/mg of protein. Dimethylformamide (8 μl) was added to 1 ml of a control sample of submitochondrial particles. The submitochondrial particles were stored at 0° for 18 hr. The submitochondrial particles were then diluted to 2 mg of protein/ml with the suspension medium. Either 0.05- or 0.1-ml aliquots of the diluted submitochondrial particle suspension were then added to either 0.95 or 0.9 ml of the reaction mixture (see Methods section), which contained 4.9 mμmoles of potassium, [^{32}P]P_i (68,400 cpm/μmole), and other additions as shown. The reaction was stopped after 10 min. ^{32}P incorporation into adenine nucleotide and the ATP content of the reaction mixture were assayed. The ATP contents were unchanged in the reaction mixtures to which DCCD-treated submitochondrial particles had been added. In the control assays, the ATP contents were approximately 0.7 μmole.

(1.1–1.2 mμmoles of DCCD/mg) of protein is required to inhibit the ATP-driven pyridine nucleotide transhydrogenase activity.

Stimulation by DCCD and Oligomycin A of the Ability of EDTA Particles to Catalyze the ATP-Driven Partial Reactions of Oxidative Phosphorylation. Lee and Ernster (1966) have shown that concentrations of oligomycin, lower than those which inhibit the ATP-driven partial reactions catalyzed by submitochondrial particles, will stimulate the ability of EDTA particles to catalyze the ATP-driven reduction of NAD^+ by succinate. Van Dam and Ter Welle (1966) have shown a similar stimulation by oligomycin of the ATP-driven pyridine nucleotide transhydrogenase in a Keilin and Hartree heart muscle preparation. The data illustrated in Figure 8 show that preincubation of EDTA particles with low concentrations of DCCD also results in a stimulation of the ATP-driven reduction of NAD^+ by succinate. The stimulation is maximal at 0.2 mμmole of DCCD/mg of protein. At higher DCCD concentrations the reaction is inhibited. It can be seen that maximum stimulation by oligomycin A is achieved at concentrations very similar to the optimum DCCD concentration. While the optimum concentrations for both DCCD and oligomycin A varied with different preparations of EDTA particles, oligomycin A invariably gave a two- to fourfold better stimulation than DCCD of either the ATP-driven reduction of NAD^+ by succinate or of the ATP-driven pyridine nucleotide transhydrogenase.

Discussion

The results presented here show that DCCD is a potent and specific inhibitor of one of the intermediate reactions leading to the synthesis of ATP, concomitant with the flow of electrons along the mitochondrial electron-transport system.

The potency of DCCD as an inhibitor of oxidative phosphorylation is illustrated in most of the experiments presented here. The preincubation of mitochondria or submitochondrial particles with 2 (or less) mμmoles of DCCD/mg of protein has a permanent effect on the reactions studied. Thus the following enzyme systems are inhibited: the stimulation of respiration in tightly coupled mitochondria by ADP (Figure 1) or by arsenate (Figure 2), the synthesis of ATP by submitochondrial particles (Table I), the ATP-driven reduction of NAD^+ either by succinate (Table I, Figure 7) or TMPD plus ascorbate (Table II, Figure 7), the ATP-driven energy-linked pyridine nucleotide transhydrogenase (Table I, Figure 7), the inherent and dinitrophenol-stimulated adenosine triphosphatase activities (Table IV), and the P_i–ATP-exchange activity (Table V). In contrast the aerobic energy-linked pyridine nucleotide transhydrogenase activity of submitochondrial particles is stimulated by these concentrations of DCCD (Table III).

The results illustrated in Figure 7 are a more precise measure of the inhibitory concentrations of DCCD. Eight-tenths to nine-tenths millimicromole of DCCD per milligram of protein is sufficient to inhibit the ATP-driven reduction of NAD^+ either by succinate or by TMPD plus ascorbate. The ATP-driven pyridine nucleotide transhydrogenase requires 1.1–1.2 mμmoles of DCCD/mg of protein for complete inhibition. The concentration of cytochrome c_1 in submitochondrial particles is 0.27 mμmoles/mg of protein (Blair *et al.*, 1963). Thus three or four molecules of DCCD per unit of electron-transport chain will inhibit completely

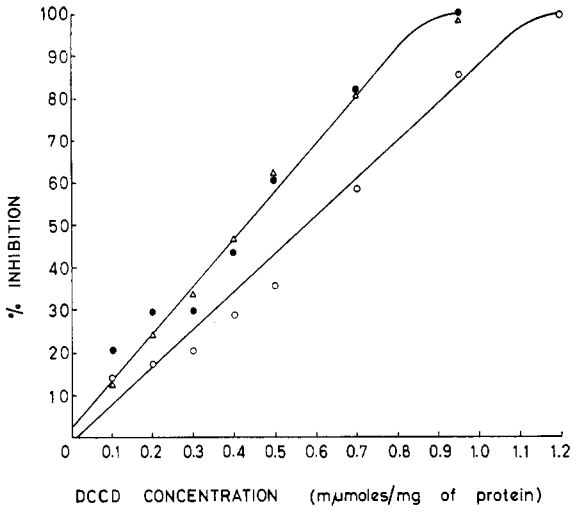


FIGURE 7: Titration of submitochondrial particles with DCCD. Submitochondrial particles were suspended in a solution containing 250 mM sucrose, 4 mM magnesium sulfate, 1 mM K-ATP, and 10 mM Tris-sulfate (pH 7.4). The final protein concentration was 16.6 mg/ml. Aliquots of ethanolic solutions of DCCD were added to samples of the suspension to give the indicated concentrations of DCCD. The final ethanol concentration in all cases was 5 μl/ml of submitochondrial particle suspension. The submitochondrial particles were incubated for 18 hr at 0°. The ATP-driven reduction of NAD^+ by succinate (△), ATP-driven reduction of NAD^+ by TMPD plus ascorbate (●), and the ATP-driven pyridine nucleotide transhydrogenase (○) activities were then assayed.

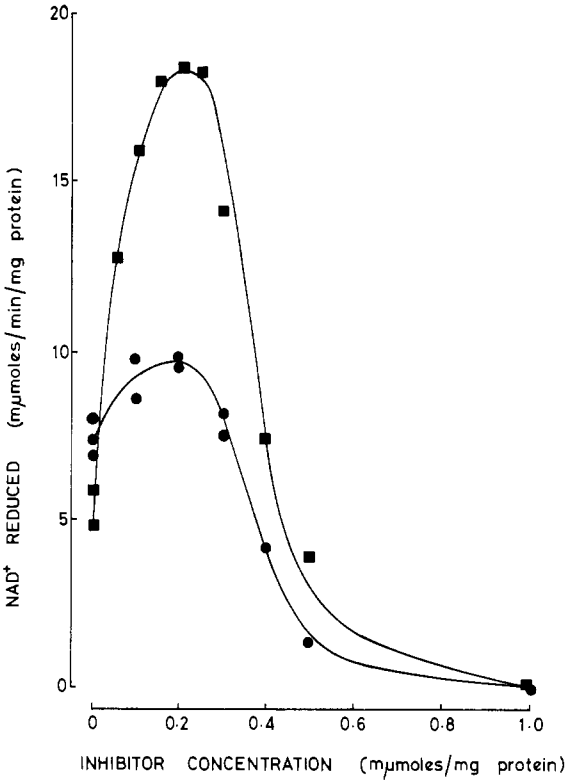


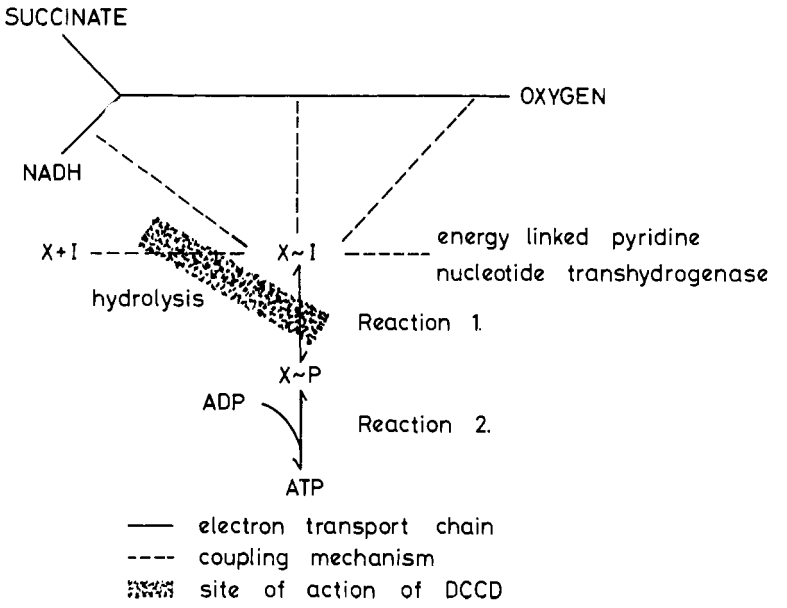
FIGURE 8: Effect of low concentrations of DCCD and oligomycin on the ATP-driven reduction of NAD^+ by succinate in EDTA particles; 1-ml samples of EDTA particles were treated with the indicated concentrations of DCCD (dissolved in 2 μl of dimethylformamide) and stored at 0° for 18 hr before assay. Oligomycin A dissolved in 7.5 μl of ethanol was added to the assay system immediately prior to the addition of EDTA particles. ATP was added 3 min later. The assay was as described in the Methods section save that the concentration of magnesium was 2 mM. (●) DCCD-treated EDTA-particles; (■) oligomycin A treated EDTA particles.

these ATP-utilizing systems. Hence, on a concentration basis DCCD acts in a very specific manner, probably at the same site in each of the phosphorylating chains. We have shown previously that DCCD inhibits the synthesis of ATP at the three known sites associated with the electron-transport chain (Beechey *et al.*, 1966).

The DCCD-sensitive site must lie in the phosphorylating systems associated with the electron-transport chain for the following reasons. All of the reactions listed above which are inhibited by DCCD involve the synthesis or utilization of ATP, but three of them do not involve the participation of the electron-transport chain: these are the reaction systems responsible for ATP–P_i exchange, adenosine triphosphatase activity, and the ATP-driven pyridine nucleotide transhydrogenase activity. The treatment of mitochondria with DCCD only results in the inhibition of electron transport when there is a functional association between electron transport and the synthesis of ATP, *i.e.*, in coupled mitochondria. The dissociation of phosphorylation from electron transport, by the addition of an uncoupling agent to a DCCD-inhibited mitochondrial preparation, results in a rapid rate of respiration which is faster than the rate of ADP-stimulated respiration in a control experiment (see Figure 1). Also, the electron-transport chain in such uncoupled DCCD-treated mitochondria is capable of organized action, as is shown in Figure 3 where the respiration rate is still controlled by the availability of ADP when α-oxoglutarate is the sole substrate. Thus, DCCD has no direct inhibitory action on the electron-transport chain.

X~I is a hypothetical intermediate in the phosphorylating chains, which is envisaged as supplying energy for the pyridine nucleotide transhydrogenase activity (Danielson and Ernster, 1963) (Scheme I). The DCCD-sensitive site must lie on the ATP side of X~I because DCCD treatment of submitochondrial particles does not result in an inhibition of the aerobic energy-linked pyridine nucleotide transhydrogenase;

SCHEME I: Relationship of Postulated Site of Action of DCCD to the Electron-Transport System Chain and Energy-Utilizing Systems.



it is in fact stimulated (Table III) by DCCD concentrations which inhibit the ATP-driven partial reactions. A DCCD-sensitive site at one of the reactions between X~I and ATP would account for the inhibition of the ATP–P_i exchange and the adenosine triphosphatase activities. Both these reactions are mediated by enzyme systems located in the (X~I)–ATP region of the phosphorylating chain. Also, such a site of action would account for the inhibition of ATP synthesis and the ATP-utilizing systems. These conclusions regarding the site of action of DCCD are summarized in Scheme I.

The effects of DCCD reported here can be achieved either by inhibiting reaction 1 or by inhibiting reaction 2 of Scheme I. In either of these inhibitory mechanisms DCCD must not modify X in such a manner as to prevent X from reacting with I to form X~I.

Inhibition of Reaction 1. If X is a compound which has a P_i binding site, or if X is a functional group on a molecule where I and the P_i binding site are functional groups adjacent to X, then the following modes of action for DCCD are possible. (i) DCCD can react at and modify chemically the P_i binding site, thus preventing the interaction of P_i with X~I. (ii) DCCD can react at some site distant from the P_i binding site and yet inhibit P_i binding by an allosteric mechanism. (iii) DCCD can combine in such a manner that either P_i binding is sterically hindered or bound P_i is sterically hindered from reacting with X~I.

Inhibition of Reaction 2. Here DCCD could inhibit by modifying one of the sites, either on X or the compound containing X as a functional group, which is involved in the reaction of X~P (X~As) with activated ADP. Pullman *et al.* (1960) have shown that F_1 will bind ADP; hence F_1 may be considered as the ADP activator in ATP synthesis. Reaction 2 may therefore be written

$$ADP(F_1) + X\sim P \rightleftharpoons ATP + F_1 + X$$

Since CF_0 (the factor conferring oligomycin sensitivity on F_1 adenosine triphosphatase activity) combines with F_1 (Kagawa and Racker, 1966), the assumption is tentatively made that CF_0 contains X. Since it has been shown that CF_0 is modified by DCCD, then DCCD may act in either of two ways: (1) by preventing $ADP(F_1)$ from combining with X, or (2) by preventing $ADP(F_1)$ from reacting with the P_i binding site on X. Roberton *et al.* (1966) have shown that F_1 combines with CF_0 treated with DCCD. This suggests that the first inhibitory mechanism is unlikely. However, if the binding between CF_0 and F_1 involves more than one site, it is possible that modification of a single binding site is insufficient to prevent the binding of F_1 by CF_0 but adequate to prevent the interaction of X~P and activated ADP. This site of action is favored by Lardy *et al.* (1964).

In DCCD-inhibited submitochondrial particles the potential energy available in X~I (generated by the oxidation of succinate) can be used to drive the pyridine nucleotide transhydrogenase reaction. If DCCD can prevent the free energy of X~I from being dissipated by other wasteful hydrolytic reactions, then the fact that the aerobic energy-linked pyridine nucleotide transhydrogenase reaction is faster in DCCD-treated submitochondrial particles than in the control submitochondrial particles may be accounted for.

The stimulation by very low concentrations of DCCD

of the ATP-driven partial reactions (see Figure 8) catalyzed by EDTA particles is most easily explained by assuming that DCCD preferentially prevents access of water to either reaction 1 or reaction 2. This will inhibit the hydrolysis of either X∼I or X∼P, thus stimulating the ATP-driven partial reactions. The precise mechanism of this action of DCCD is obscure.

There are several observations which when considered together suggest that covalent bonds are formed at the DCCD-sensitive site: the slowness of the onset of inhibition (see Figures 4–6). The possibility that the time required for complete inhibition by DCCD is a function of some permeability barrier is made unlikely by the fact that this time requirement is common for the onset of DCCD inhibition in both intact mitochondria and in submitochondrial particles, the latter being everted fragments of the inner membrane of mitochondria (Lee and Ernster, 1966). The temperature coefficient of the inhibitory effect is approximately 2 (see Figure 5). This is a figure characteristic of a process involving the formation or breaking of covalent bonds. The first step of many of the reactions involving carbodiimides is considered to be a protonation of one of the imine nitrogen atoms (Khorana, 1961). Such a mechanism will be favored by an increased concentration of protons. The inhibitory effects of DCCD are not reversed (Holloway *et al.*, 1966) by treatments which reverse the inhibitory effects of oligomycin (Kagawa and Racker, 1966). Also the inhibitory effects of DCCD are still manifest in the properties of CF_0 and CF_0F_1 preparations (Kagawa and Racker, 1966) which have been isolated from either DCCD-treated mitochondria or DCCD-treated mitochondrial fragments (Roberton *et al.*, 1966). The inhibitory properties of DCCD are possessed by other carbodiimides, *e.g.*, diisopropylcarbodiimide, di-*p*-tolylcarbodiimide, and dibenzylcarbodiimide. This observation indicates that the diimide moiety of DCCD is responsible for the effects on oxidative phosphorylation (I. G. Knight, R. B. Beechey, A. M. Roberton, and C. T. Holloway, unpublished data). It is possible to extract all the radioactivity from [^{14}C]DCCD-treated submitochondrial particles. Thin layer chromatography shows that some of the extracted ^{14}C is incorporated in a molecule which is neither DCCD nor dicyclohexylurea (C. T. Holloway, I. G. Knight, R. B. Beechey, and A. M. Roberton, unpublished data).

It is of interest to consider the functional relationships of DCCD with the other compounds which are thought to inhibit electron transport in coupled mitochondria by acting at sites in the ATP-synthesizing system. The presently known inhibitors can be classified into two groups on the basis of two properties: (a) the site of action within the ATP-synthesizing system and (b) the specificity of the inhibitor for one preferred site or all those sites of ATP synthesis. The first group includes the alkylguanidines (Pressman, 1963a; Chappell, 1963), amytal (Chance and Hollunger, 1963), 2-alkyl-4-hydroxyquinoline *N*-oxides and 2-hydroxy-3-(3-methylbutyl)-1,4-naphthoquinone (hydrolapachol) (Howland, 1963), phenethylbiguanide (Pressman, 1963b), and decamethylenediguanide (Guillory and Slater, 1965). All these authors have interpreted their results in terms of the inhibitor acting at the level of a nonphosphorylated intermediate and the inhibitor competing with uncoupling agents for the intermediate. However, Mitchell (1966) has suggested some alternative modes of action based on: (a) the known capacity of some of these compounds to inhibit electron transport, (b) the relative lipophilicity of the inhibitors and uncoupling agents, and (c) the cationic nature of some of these inhibitors and the ability of uncoupling agents to discharge the membrane potential of the inner mitochondrial membrane, thus causing the release of these charged electron-transport inhibitors. DCCD does not belong to this group, since there is no apparent competition between uncoupling agents and DCCD; neither does DCCD show specificity in inhibiting phosphorylation associated with one particular site, nor does DCCD inhibit uncoupled electron transport.

The second group of inhibitors includes the oligomycins A–C (Lardy *et al.*, 1958), rutamycin (oligomycin D) (Lardy *et al.*, 1965), and aurovertin (Lardy *et al.*, 1964). These compounds inhibit energy transfer at the level of the phosphorylated high-energy intermediates, though not necessarily at the same site (Lardy *et al.*, 1964). Uncoupling agents reverse immediately the inhibition of electron transport by these compounds in tightly coupled mitochondria. DCCD obviously belongs to this group of inhibitors. The site of action of DCCD indicated in Scheme I is identical with that suggested for oligomycin by Ernster and Lee (1964).

There are many similarities between the effects of DCCD and those of oligomycin. Thus oligomycin inhibits: (1) the coupled respiration of intact mitochondria and this inhibition is relieved by uncoupling agents but not by arsenate; (2) the uncoupling agent stimulated adenosine triphosphatase activity of mitochondria and submitochondrial particles; (3) P_i–ATP-exchange activity; (4) the ATP-driven reduction of NAD^+ either by succinate or by TMPD plus ascorbate; and (5) the ATP-driven energy-linked pyridine nucleotide transhydrogenase system. Oligomycin stimulates the aerobic energy-linked pyridine nucleotide transhydrogenase system and at very low concentrations the ATP-driven reduction of NAD^+ by succinate in EDTA particles.

There are several observations which differentiate the mode of action of DCCD from that of oligomycin. The rates of onset of inhibition are very different. Inhibition by oligomycin is almost immediate while DCCD requires 0–24 hr to manifest its action, depending on the pH, temperature, and concentration of DCCD. Oligomycin is more efficient than DCCD in stimulating the ATP-driven reduction of NAD^+ by succinate (Figure 8). Also the action of DCCD on CF_0F_1 (*i.e.*, to inhibit the phospholipid stimulation of the adenosine triphosphatase activity of CF_0F_1) is irreversible (A. M. Roberton, unpublished results) under conditions which reverse the inhibitory effect of rutamycin (oligomycin D) (Kagawa and Racker, 1966). Similarly, the adenosine triphosphatase activity of

DCCD-treated submitochondrial particles is not restored by phospholipid washing (Holloway *et al.*, 1966), in contrast to rutamycin-treated submitochondrial particles in which the activity is restored by phospholipid washing (Kagawa and Racker, 1966). A comparison of the inhibitory actions of oligomycin and aurovertin has been made by Lardy *et al.* (1964); the points of similarity and difference apply with equal force to DCCD.

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