INDUCTION OF RESPIRATORY CONTROL IN SUBMITOCHONDRIAL PARTICLES BY DICYCLOHEXYLCARBODIIMIDE

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Beechey et al. (1966) have reported that DCCD* acts as an inhibitor of oxidative phosphorylation in a fashion similar to that of oligomycin and aurovertin. These workers (Holloway et al., 1966; Roberton et al., 1966) have also reported that DCCD binds irreversibly to the F_o fraction isolated by Kagawa and Racker (1966). Racker and Horstman (1967) have shown recently that DCCD, at low concentration, may increase the rate of phosphorylation in submitochondrial particles. This communication reports some of our observations on respiratory control induced by DCCD in ETPH(Mg^{++},Mn^{++}), as well as the effects of this inhibitor on ATPase and electron transfer components.

Methods. Submitochondrial particles from beef heart mitochondria were prepared according to Beyer (1967). Respiration was measured with the Clark electrode at 25° in an enclosed glass

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*Abbreviations: DCCD, dicyclohexylcarbodiimide; ETPH(Mg^{++},Mn^{++}), phosphorylating submitochondrial particles; ETPH(EDTA-2), poorly phosphorylating submitochondrial particles; EPR, electron paramagnetic resonance; F_3CCP, paratrifluoromethoxycarbonylcyanide phenylhydrazone; DNP, 2,4-dinitrophenol; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; EDTA, ethylenediaminetetraacetate; FeNH, non-heme iron proteins, the subscripts D, S, and R referring to the DPNH dehydrogenase, succinate dehydrogenase, and the cytochrome b-c_1 complex respectively with which they are associated.

cuvette. Inorganic orthophosphate was determined by the isobutanol-benzene extraction method as described by Lindberg and Ernster (1956). Steady state oxido-reduction levels of cytochromes and flavin were observed in the Aminco-Chance dual wavelength spectrophotometer. EPR spectra were obtained with a Varian X-band spectrometer.

Results and Discussion

DCCD and rate of respiration. The addition of 267 millimicromoles DCCD per mg protein to ETPH(Mg^{++},Mn^{++}) oxidizing NADH re-

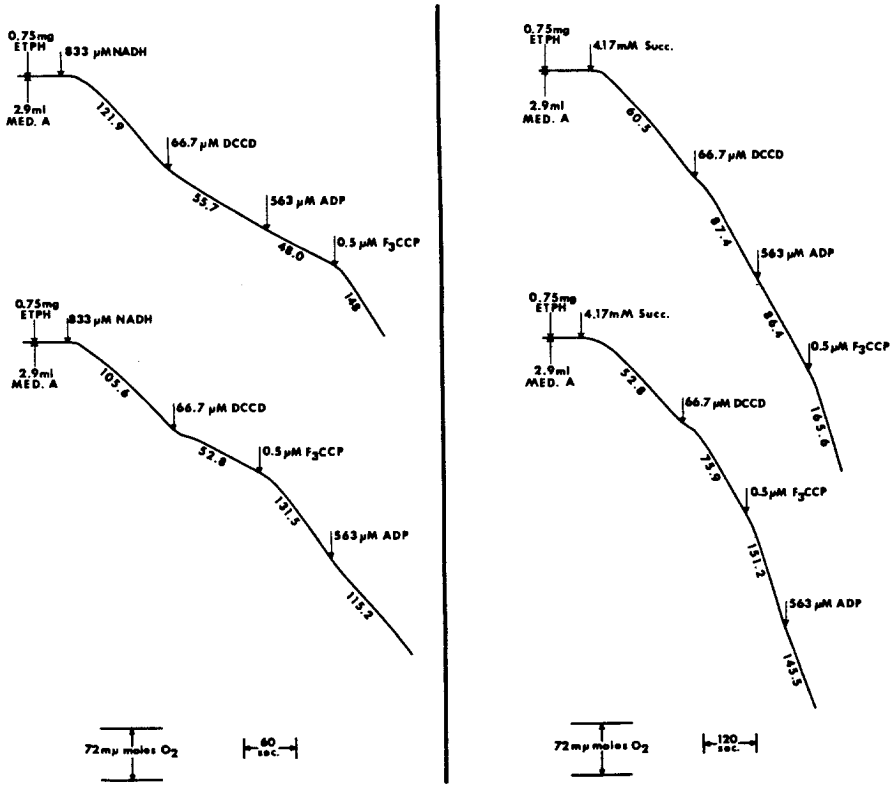

Fig. 1. The effect of DCCD on respiration of ETPH(Mg^{++},Mn^{++}). The lines represent tracings from the oxygen electrode. All measurements were made at oxygen saturations greater than 30% that of air at 25°. The numbers immediately below the tracings refer to the rate of oxygen consumption in millimicromoles per minute per mg protein. Medium A contained 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 5 mM K_2HPO_4, pH 7.5. DCCD and F_3CCP were added as ethanolic solutions in 0.005 ml volumes.

sulted in an immediate inhibition of oxygen consumption to approximately half the original rate (Fig. 1). The inhibition was not overcome by ADP at the concentration used in Fig. 1 or at higher concentrations. F_3CCP relieved the inhibition conferred by DCCD, as did DNP and tyrocidin. The addition of ADP during the uncoupler-released state also did not significantly affect the respiratory rate. Contrary to its effect on NADH oxidation, DCCD stimulated succinate oxidation by ETPH(Mg^{++},Mn^{++}) (Fig. 1). Once again, ADP was without significant effect on succinate oxidation when added either before or after F_3CCP. This uncoupler of oxidative phosphorylation increased the rate of succinate oxidation whether added in the presence or absence of ADP. These data indicate that the site of inhibition of DCCD exerted upon the electron transfer chain resides between NADH and the first component common to both the NADH and succinate branches. Preliminary studies of the effect of DCCD on the NADH-TMPD and succinate-TMPD shunts (Lee et al., 1965) over the antimycin A sensitive site indicated that the inhibitor was without effect in such systems. In addition, DCCD had little or no effect on the rate of oxidation of cytochrome c reduced by ascorbate in the presence of antimycin A even at levels of DCCD greater than 2 micromoles per mg particle protein.

Effect of DCCD on ATPase catalyzed by ETPH(Mg^{++},Mn^{++}). Beechey et al. (1966) have reported that DCCD inhibits the DNP-stimulated ATPase of intact rat heart mitochondria in some non-competitive manner. DCCD also inhibited the ATPase catalyzed by ETPH(Mg^{++},Mn^{++}) (Table 1). Essentially complete inhibition was obtained at 20 millimicromoles of DCCD per mg protein while half maximal inhibition was obtained at 4 millimicromoles DCCD

per mg protein. DCCD inhibited the ATPase in the presence of optimal DNP (10^{-3} M) and in the absence of the uncoupler to approximately equal extents; for example, 4 millimicromoles DCCD lowered both ATPase conditions by about 50%. Higher concentrations of DNP inhibited ATPase. At concentrations of

Table 1. Effect of DCCD on ATPase of ETPH(Mg^{++},Mn^{++})

DNP	P_i released (micromoles/min/mg protein)		
	No DCCD	DCCD 4 mμmoles/mg prot.	DCCD 20 mμmoles/mg prot.
None	0.84	0.39	0.08
10^{-4} M	0.86	0.49	0.11
10^{-3} M	1.23	0.63	0.07
4 x 10^{-3} M	0.84	0.40	0.06

Each assay tube contained 5 micromoles $MgCl_2$, 50 micromoles Tris·HCl, pH 7.5, and 0.1 mg ETPH protein, in addition to those compounds listed in the table, in a volume of 0.96 ml. After 3 minutes equilibration at 25°, 0.06 ml of 0.1 M ATP was added to initiate the reaction. The reaction was terminated after 10 minutes and the P_i released from ATP was corrected for "no enzyme" blanks.

DCCD which essentially completely inhibited ATPase, DNP was without significant effect. These data clearly indicate that DNP does not release the inhibition imposed by DCCD and suggest that only that portion of the ATPase which has not reacted with DCCD may respond to DNP.

Effect of DCCD on the respiratory chain. The effect of DCCD, at a concentration which caused 30% inhibition of NADH oxidation, on the components of the electron transfer chain has been studied in an attempt to ascertain the point of inhibition (Table 2). The addition of NADH to the submitochondrial particles results in a partial reduction of the components. The extreme state of reduction of cytochrome *b* may be a result of the known deficiency of cytochrome *c* in sonic submitochondrial particles. During the DCCD-inhibited state flavin

shifted toward further reduction while cytochrome b became more oxidized, indicating that a crossover point existed between these two components under the influence of DCCD. The uncoupler, F_3CCP, which partially relieved the DCCD-induced inhibition, caused a small shift toward reduction of flavin which may re-

Table 2. Effect of DCCD on Steady-State Oxidation-Reduction Levels of Cytochromes and Flavin

Additions	% reduction				
	Flavin 465-510	Cyt. b 564-575	Cyts. c + c_1 550-541	cyt. a 655-630	cyt. a_3 445-460
NADH	47	93	21	18	20
DCCD	78	76	31	20	21
F_3CCP	84	36	44	33	23

The numbers directly under the components refer to the wavelength pair used to monitor each component in the Aminco-Chance dual wavelength spectrophotometer. The extinction coefficients employed to calculate concentrations of components were those recommended by Chance and Williams (1956) except for cytochromes a and a_3 for which the corrected values of Van Gelder (1966) were used. Reactions were measured at room temperature in cuvettes of 10 mm path length containing in 3 ml the following components: 2.9 ml of a medium containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 5 mM K_2HPO_4, pH 7.5; 1.5 mg ETPH(Mg^{++},Mn^{++}) protein. The concentrations of other constituents were those reported in Fig. 1.

flect the reduction of succinic dehydrogenase flavin. The uncoupler also caused a further shift toward oxidation of cytochrome b, the same direction when F_3CCP is added to such particles in the absence of DCCD.

The effect of DCCD on non-heme iron proteins of the electron transfer chain was investigated in a preliminary experiment in the EPR spectrometer. The particle used in this experiment was ETPH(EDTA-2) which avoids the use of Mn^{++} during its preparation (Beyer, 1967). This metal obscures the EPR signals of iron and copper in the electron transfer chain. Incubation of ETPH(EDTA-2) with NADH for 30 seconds at room temperature results in the appearance of the characteristic g=1.94 signals

of non-heme iron associated with NADH dehydrogenase, succinate dehydrogenase, and the cytochrome b-c_1 complex (Beinert, 1965). When particles which had previously been treated with DCCD so as to inhibit the oxidation of NADH by over 90% were so incubated, only the characteristic signal for the $FeNH_D$ was observed. An additional two minutes of incubation at room temperature did not elicit the $FeNH_S$ or $FeNH_R$ signals. These preliminary data allow us to further narrow the point of inhibition by DCCD of electron transfer to a site between $FeNH_D$ and cytochrome b.

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