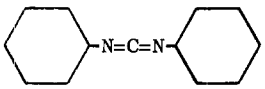
DICYCLOHEXYLCARBODIIMIDE - AN INHIBITOR OF OXIDATIVE PHOSPHORYLATION

R. B. Beechey, Caroline T. Holloway[1], I. G. Knight and A. M. Roberton.

"Shell" Research Ltd., Milstead Laboratory of Chemical Enzymology, Broad Oak Road, Sittingbourne, Kent, United Kingdom.

Received March 2, 1966

This communication reports some of the properties of a new inhibitor of oxidative phosphorylation, DCCD*, whose mode of action is similar to oligo-



mycin and aurovertin (Lardy et al., 1958). Low titres of DCCD inhibit the coupled respiration of mitochondria and the ATP-driven partial reactions of oxidative phosphorylation catalysed by ETP_H.

Methods. Mitochondria were prepared by the following methods; ox heart mitochondria (Sanadi & Fluharty, 1963), rat heart mitochondria (Chance & Hagihara, 1961) and rat liver mitochondria (Schneider & Hogeboom, 1950). ETP_H were prepared by the method of Hansen & Smith (1963) omitting manganese from the sonication mixture. Respiration rates were measured with an oxygen electrode (Chappell, 1961).

Results and Discussion

The effect of DCCD on the coupled respiration of mitochondria. The effect of DCCD on mitochondrial respiration is shown in Fig. 1, with ascorbate + TMPD (experiment A), glutamate + malate (experiment B) and succinate in the presence of rotenone (experiment C) as substrates. In all experiments ADP was first

---

1. Milstead Research Fellow.

*Abbreviations: DCCD, dicyclohexylcarbodiimide; ETP_H, phosphorylating submitochondrial particles; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; DNP, 2,4-dinitrophenol; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

added to establish that the mitochondria exhibited respiratory control. The addition of DCCD had little effect on the State 4 respiration rate though occasionally this rate was slightly enhanced. In experiments A and B, when

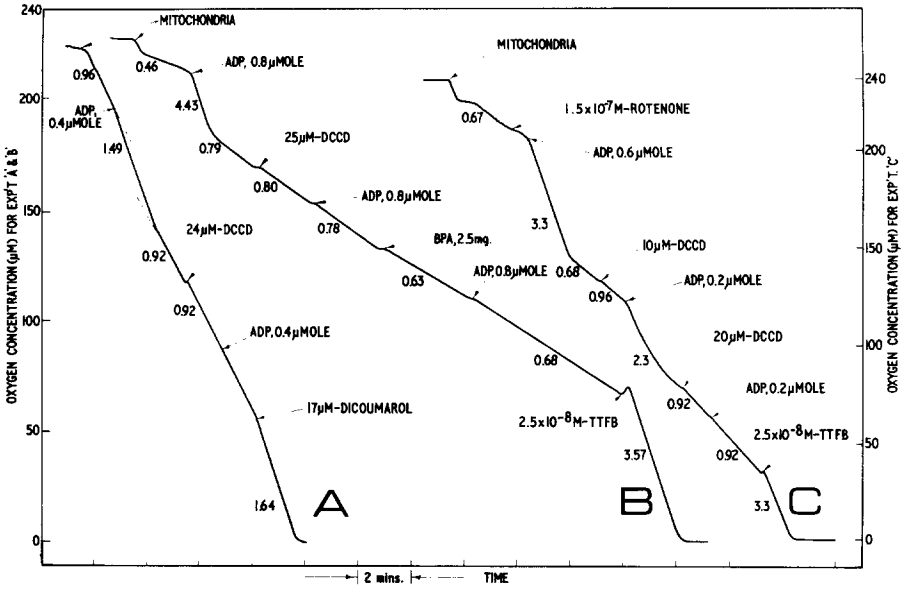


Fig. 1. The effect of DCCD on the coupled respiration of mitochondria. The lines represent the output from an oxygen electrode. The numbers on the lines are respiration rates, μmoles of oxygen $mg.^{-1}$ protein $hr.^{-1}$ at 25°. Experiment A: rat heart mitochondria (0.7 mg. protein) were added to a reaction mixture consisting of 4.1 ml. of 0.25M-sucrose containing 1mM-EDTA, 20μl. of 0.5M-ascorbate, 10μl. of 0.1M-TMPD and 40μl. of 1M-phosphate, pH 7.4. Experiment B: ox heart mitochondria (3 mg. of protein) were added to a reaction mixture consisting of 3.8 ml. of 0.25M-sucrose containing 1mM-EDTA, 20μl. of 1M-malonate, 10μl. of 1M-glutamate, 10μl. of 1M-DL-malate, 40μl. of 1M-$MgCl_2$ and 30μl. of 1M-phosphate, pH 7.4. Experiment C: rat heart mitochondria (0.7 mg. protein) were added to a reaction mixture consisting of 3.8 ml. of 0.25M-sucrose containing 1mM-EDTA, 40μl. of 1M-succinate and 30μl. of 1M-phosphate, pH 7.4.

25μM-DCCD was present, the subsequent addition of ADP did not increase the respiration rate, i.e. State 3 respiration was prevented. In experiment C, when 10μM-DCCD was present, the second addition of ADP caused an increase in the respiration rate, but increasing the DCCD concentration to 30μM prevented the stimulatory effect of a subsequent addition of ADP. In experiment B the consecutive additions of bovine plasma albumin and ADP showed that the inhibition of State 3 respiration by DCCD was not reversed by bovine plasma albumin.

The addition of uncoupling concentrations of TTFB (Beechey, 1965) in experiments B and C and of dicoumarol in experiment A stimulated the respiration to a rate which was not less than 80% of the initial State 3 rate and frequently was faster. The uncoupling agents DNP and tribromoimidazole (Beechey, 1965) acted in a similar manner.

The amount of DCCD required to inhibit completely the onset of State 3 respiration appears to vary with both the amount of mitochondrial protein present and the time of pre-incubation of DCCD with the mitochondria in State 4. The presence of DCCD at concentrations greater than 40 mμmoles DCCD/mg. mitochondrial protein immediately prevented the start of a State 3 respiration rate by ADP. However, 15 mμmoles DCCD/mg. protein inhibited the onset of a State 3 respiration rate when the mitochondria were pre-incubated with DCCD in State 4 for 10 mins. This time factor and the fact that DCCD is quite rapidly hydrated to dicyclohexylurea (Khorana, 1953), make it difficult to assess the minimum titre of DCCD required to inhibit State 3 respiration. These results apply equally to mitochondria isolated from rat heart and liver and ox heart.

Effect of DCCD on partial reactions of oxidative phosphorylation in ETP_H.

Energy-linked pyridine nucleotide transhydrogenase. The reduction of $NADP^+$ by NADH with ATP as the energy source (Danielson & Ernster, 1963) was inhibited immediately and completely by high concentrations of DCCD (see Table 1). In contrast similar concentrations of DCCD were without effect on the reaction when driven by energy produced during the oxidation of succinate (Table 1). The energy-linked transhydrogenase driven by succinate oxidation was 68% inhibited by the addition of 100μM-DNP. The subsequent addition of DCCD increased the degree of inhibition to 82%. Control assays showed that the reaction rate was linear over the reaction period of the former experiment.

ATP-dependent reduction of NAD^+ by succinate (Löw et al., 1961). 200 mμmoles DCCD/mg. protein immediately inhibit this reaction by 100%.

The minimum DCCD titre which will inhibit the ATP-dependent trans-

Table 1. Effect of DCCD on the pyridine nucleotide transhydrogenase reactions catalysed by submitochondrial particles.

Rate of $NADP^+$ reduction (mμmoles $mg.^{-1}$ protein min^{-1})			
Experiment	I	II	III
Energy source	Succinate + O_2		ATP
Control	152	160	164
+DCCD (150 mμmoles/mg. protein)	145	-	5
+DNP (100 μM)	-	51	-
+DCCD + DNP	32	30	-

The reaction mixture contained: 250mM-sucrose; 5mM-$MgCl_2$; 50mM-Tris-HCl, pH 8.0; 400mM-ethanol; 66μM-NAD^+; 150 μg. yeast alcohol dehydrogenase; 0.66μM-rotenone; ETP_H, 0.66 mg. protein; 0.33mM-$NADP^+$ and either 1.33mM-succinate or 1mM-KCN and 1.33mM-ATP. Final volume 3 ml., temperature 30°. The change in extinction at 340mμ was followed in a Zeiss PMQ II spectrophotometer.
Experiment I. The control rate was measured separately. DCCD and ETP_H were added at zero time, succinate at 1 min, $NADP^+$ at 2 min and DNP at 4 min.
Experiment II. ETP_H was added at zero time, succinate at 1 min, $NADP^+$ at 2 min, DNP at 4 min and DCCD at 6 min.
Experiment III. The control rate was assayed separately. KCN, ETP_H and DCCD were added at zero time, $NADP^+$ at 1 min and ATP at 2 min.

hydrogenase, the ATP-dependent reduction of NAD^+ by succinate and the ATP-driven reduction of NAD^+ by TMPD + ascorbate (Löw et al., 1963) depends on the length of pre-incubation of DCCD with ETP_H before starting the reaction. In the experiment illustrated in Fig. 2, DCCD was preincubated with ETP_H in the reaction medium for 10 mins. before the reaction was started. 50% inhibition of the ATP-driven transhydrogenase was noted at 9 mμmoles DCCD/mg. protein; the corresponding figure for oligomycin A was 0.7 mμmoles/mg. protein. The ATP-driven reduction of NAD^+ by succinate was 50% inhibited by 5 mμmoles DCCD/mg. protein and 0.3 mμmoles of oligomycin/mg. protein.

Effect of DCCD on the DNP-stimulated ATP-ase activity of mitochondria. The DNP-stimulated ATPase is inhibited by DCCD. This is shown by the experiment illustrated in Fig. 3 where the capacity of 10, 30 and 100μM-DNP to stimulate mitochondrial ATPase activity was measured in the presence of 10, 50 and 100μM-DCCD. From the reciprocal plot illustrated in Fig. 3 it would appear that there is some form of non-competitive inhibition by DCCD on the DNP-stimulated

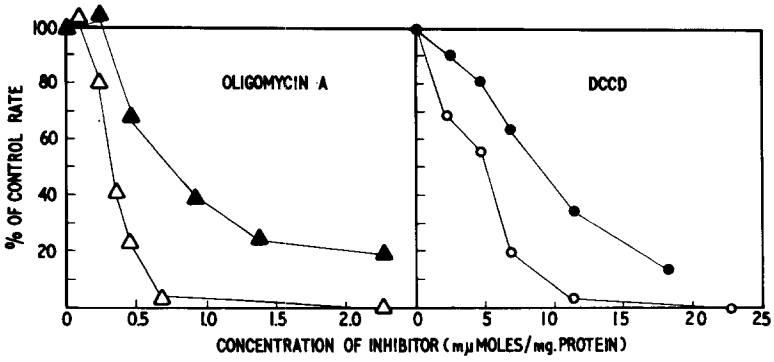


Fig. 2. Inhibition of partial reactions of oxidative phosphorylation. ATP-linked pyridine nucleotide transhydrogenase activity was measured in the presence of oligomycin A (▲—▲) and DCCD (●—●) essentially as described in Table I., except that the inhibitors were pre-incubated with ETP_H for 9 min. before $NADP^+$ addition and ATP was added at 10 min. Control rate 227 mμmoles $NADP^+$ reduced $min.^{-1}$ $mg.^{-1}$ protein. ATP-linked reduction of NAD^+ by succinate was measured in the presence of oligomycin A (△—△) and DCCD (○—○) essentially as described by Griffiths & Roberton (1965), except that inhibitors were pre-incubated with ETP_H for 10 min. before ATP addition. 0.45 mg. protein were used in each experiment. Control rate 194 mμmoles NAD^+ reduced $min.^{-1}$ $mg.^{-1}$ protein.

ATPase activity of mitochondria.

The results presented here show that DCCD affects respiration by acting at a specific site in the phosphorylating enzyme systems associated with the electron transport chain. The evidence for a specific site of action may be summarised as follows. Uncoupling agents relieve DCCD inhibited respiration of mitochondria. The energy-linked pyridine nucleotide transhydrogenase and the reduction of NAD^+ by reversed electron flow are inhibited only when ATP is the energy source. Unpublished results have shown that DCCD has no effect on substrate-linked phosphorylation associated with the oxidation of α-oxoglutarate. Control experiments have shown that DCCD is the active compound and not dicyclohexylurea which is the product of the combination of DCCD with water.

The mode of action of DCCD has many features in common with that of oligomycin. However, little is known of the structure and chemistry of oligomycin, whereas DCCD is a simple compound with well documented chemical

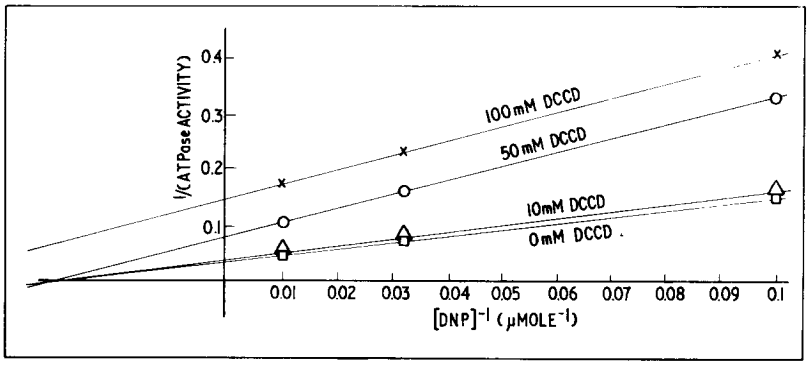


Fig. 3. The effect of DCCD concentration on the stimulation of mitochondrial ATPase activity by different concentrations of DNP. Rat heart mitochondria (1.16 mg. of protein) were suspended in a medium containing 150mM-sucrose, 2mM-ATP and 50mM-Tris-HCl, pH 7.4. 1.0 ml. samples of this reaction medium were incubated with 10μl. aliquots of DNP and DCCD, such that for each concentration of DCCD there were 3 different concentrations of DNP. After 5 mins. incubation at 20° the reaction was stopped by the addition of 0.1 ml. of 40% trichloracetic acid and the precipitated mitochondria removed by centrifugation. The orthophosphate content of the supernatant liquid was then assayed by the method of Summer (1944).

properties (Khorana, 1953). [^{14}C]DCCD has been synthesised and studies on the locus and mode of action are in progress.

REFERENCES

Beechey, R. B. Biochem. J. (1966) in press.

Chance, B. & Hagihara, B. Symp. Intracellular Respiration, Phosphorylating and Non-phosphorylating Oxidation Reactions, Proc. Intern. Congr. Biochem. 5th Moscow, 5, 3 (1961). Slater, E. C. ed., Pergammon Press, London.

Chappell, J. B. Biol. Structure and Function, 2, 71 (1961). Goodwin, T. W. and Lindberg, O. eds., Academic Press, New York.

Danielson, L. & Ernster, L. Biochem. Z. 338, 188 (1963).

Griffiths, D. E. & Roberton, A. M. Biochim. Biophys. Acta, (1966) in press.

Hansen, M. & Smith, A. L. Biochim. Biophys. Acta, 82, 200 (1964).

Khorana, H. G. Chem. Rev., 53, 145 (1953).

Lardy, H. A., Johnson, D. & McMurray, W. C., Arch. Biochem., 28, 317 (1958).

Löw, H., Krueger, H. & Zeigler, D. M., Biochem. biophys. Res. Commun., 5, 231 (1961).

Löw, H., Vallin, I. & Alm, B. In 'Energy Linked Functions of Mitochondria' p.5 (1963), Chance, B. ed., Academic Press, New York.

Sanadi, D. R. & Fluharty, A. L., Biochemistry, 2, 523 (1963).

Schneider, W. C. & Hogeboom, J. H., J. biol. Chem., 183, 123 (1950).

Summer, J. B., Science, 100, 413 (1944).