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# INHIBITION OF $K^+$ FLUX INTO RAT LIVER MITOCHONDRIA BY DICYCLOHEXYLCARBODIIMIDE

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SUMMARY: Dicyclohexylcarbodiimide (DCCD), which has been reported to react covalently with a subunit of the ATP synthase complex involved in proton translocation, decreases the rate of unidirectional $K^+$ flux into rat liver mitochondria in the presence of the respiratory substrate, succinate. In contrast, oligomycin does not affect the rate of $K^+$ influx. At concentrations which completely block ADP-stimulated respiration, DCCD increases the apparent Km for $K^+$ of the $K^+$ transport mechanism, while having little effect on the Vmax of $K^+$ influx. Inhibition of $K^+$ influx by DCCD is observed over the whole pH range tested from 6.8 to 8.0. A slight, but reproducible, decrease in the pH-dependence of $K^+$ influx is observed in the presence of DCCD.

The oxidative phosphorylation inhibitor, DCCD[1] reacts covalently with one of the hydrophobic membrane-sector subunits of the mitochondrial ATP synthase complex (1,2). The DCCD-binding proteolipid is also the site of interaction of oligomycin (3). Much evidence indicates a role of the DCCD-binding proteolipid in proton ( or hydroxyl ion) translocation. Oligomycin decreases the permeability to protons induced by dissociation of the F1 ATP-ase from mitochondrial vesicle membranes (4). The DCCD-binding subunit, or proteolipid fractions containing this subunit, isolated from yeast mitochondria (3,5), beef heart mitochondria (6,7) or lettuce chloroplasts (8), mediate proton fluxes in artificial lipid membranes which are sensitive to inhibition by oligomycin or DCCD.

Rates of unidirectional $K^+$ flux both into and out of rat liver mitochondria are dependent on respiration (9). Rates of $K^+$ flux into rat liver or beef heart mitochondria are saturable with respect to the external $K^+$ concentration (10,11). The Vmax of $K^+$ flux into rat liver mitochondria increases as the pH of the medium is increased from 7.0 to 8.0, while the apparent Km for $K^+$

[1]Abbreviations used: DCCD, N,N'-dicyclohexylcarbodiimide; NEM, N-ethyl maleimide.

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changes very little (10). It has been suggested that the pH-dependence of the kinetics of $K^+$ influx is consistent with the possibility that the energy-linked mechanism may directly couple $K^+$ influx to $OH^-$ influx (10). The mitochondrial $K^+$ transport mechanism is thus postulated to be non-electrogenic and directly associated with the mechanism of energy transduction (10).

Brierley and coworkers have suggested that the energy-dependence of $K^+$ flux rates may alternatively be explained on the basis of involvement of two passive mechanisms, a $K^+$ uniport mechanism mediating electrophoretic $K^+$ entry and a $K^+/H^+$ exchanger mediating $K^+$ efflux (12). However, the proposal that a passive $K^+/H^+$ exchanger mediates $K^+$ efflux is not supported by data showing that the rate of $K^+$ efflux under energized conditions increases with increasing external pH (13,14).

The present experiments have examined the effect of DCCD on the kinetics of unidirectional $K^+$ flux into rat liver mitochondria in the presence of the respiratory substrate, succinate.

## METHODS

Rat liver mitochondria were isolated by standard procedures (15) as in previous experiments (10). DCCD-treated mitochondria were pre-incubated at 0° C for at least 30 minutes in the presence of DCCD. Respiration was recorded with a Clark-type membrane covered oxygen electrode. For $K^+$ flux measurements, control or DCCD-treated mitochondria were incubated at 20° C in media (pH adjusted with HCl) containing 200 mM sucrose, 30 mM Tris, 7.5 mM succinate, various concentrations of KCl, $^{42}K$ (approx. 0.6 $\mu$Ci/ml), $^3H_2O$ (approx. 2.6 $\mu$Ci/ml) and ($^{14}C$)sucrose (approx. 0.4 $\mu$Ci/ml). Mitochondrial samples were separated from incubation media by rapid centrifugation through silicone (16,17). $K^+$ concentrations were determined by atomic absorption. Radioisotopes were assayed by liquid scintillation counting. Protein was measured by the biuret method (18). Volumes of internal and external fluid compartments, and the mitochondrial content of labeled $K^+$ were calculated from the data as in previous experiments (17).

Unidirectional $K^+$ influx rates were calculated as the difference in labeled $K^+$ content between samples taken after 0.75 and 7 minutes of incubation. The values of labeled $K^+$ content were not corrected for the contaminating external $K^+$, which was estimated from the measured ($^{14}C$)sucrose spaces to be essentially constant during the time-course of the measurements. During 7 minutes of incubation, the increase in labeled $K^+$ content is an essentially linear function of incubation time (10).

All radioisotopes were obtained from New England Nuclear. The silicone used (SF1154) was a gift from the General Electric Co. DCCD was obtained from Sigma Chemical Co.

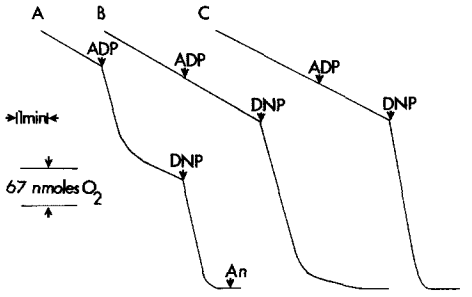



Figure 1: Effect of DCCD and oligomycin on state 3 respiration. Mitochondria (1.6 mg protein/ml) were incubated at 20°C in 2.0 ml of medium containing 200 mM sucrose, 40 mM Tris, 5 mM succinate, 5 mM $P_i$ and 2 mM KCl, adjusted to pH 7.5 with HCl. ADP (0.45 μmole) and 2,4-dinitrophenol (DNP, 0.2 μmole) were added where indicated. An indicates anaerobiosis. Trace A, control; Trace B, the medium included oligomycin (0.8 μgm/mg protein); Trace C, the mitochondria were pretreated with DCCD (36 nmoles/mg protein).

## RESULTS

The concentrations of DCCD utilized in these experiments (25-46 nmoles/mg protein) are in the range of concentrations previously reported (19) to cause immediate inhibition of ADP-stimulated respiration. Oxygen electrode recordings confirming that DCCD blocks oxidative phosphorylation under the conditions of the present experiments are presented in Figure 1. As shown in Trace C, DCCD completely prevents the stimulation of respiration by ADP in the presence of $P_i$ and succinate. Oligomycin also completely blocks state 3 respiration under the conditions tested.

Unidirectional $K^+$ influx measurements carried out under conditions of approximate steady-state $K^+$ content show no effect of oligomycin, while DCCD significantly inhibits $K^+$ influx. For example, in one experiment in which the pH of the medium was 7.5 and the external $K^+$ concentration was 2.7 mM, the $K^+$ influx rate for control samples was 0.80 $\pm$ 0.07 (5) μmoles $K^+$ per gram of protein per minute , average $\pm$ standard deviation (no. of determinations). $K^+$ influx rates determined for mitochondria treated with oligomycin (0.4 μg/mg protein) or DCCD (29nmoles/mg protein) were 0.78 $\pm$ 0.04 (4) and 0.47 $\pm$ 0.13 (6) μmoles $K^+$ per gram of protein per minute respectively.

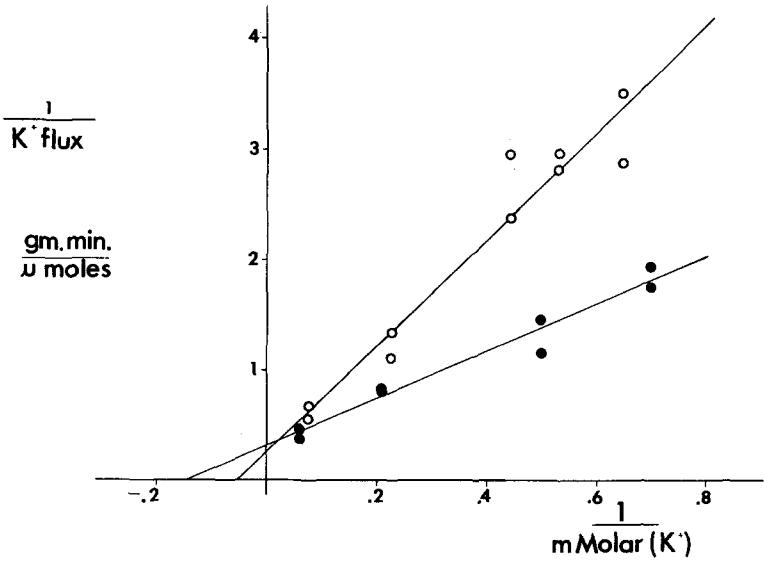
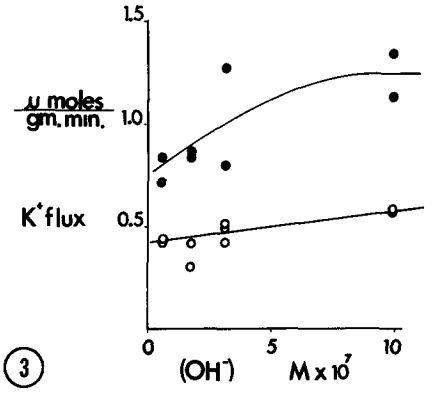



Figure 2: Effect of DCCD on the dependence of $K^+$ influx on external $K^+$ concentration. The mitochondrial protein concentration was 7.6 mg/ml. The pH of the medium was 7.5. The reciprocal of the $K^+$ influx rate, in units of gram protein (min)($\mu$moles $K^+$)$^{-1}$, is plotted against the reciprocal of the external mM $K^+$ concentration. The lines drawn are calculated by the method of least squares. Symbols: ●, control samples; ○, the mitochondria were pretreated with DCCD (26 nmoles/mg protein).

The effect of DCCD on the dependence of $K^+$ influx on external $K^+$ concentration is depicted in Figure 2. The reciprocal of the $K^+$ influx rate remains a linear function of the reciprocal of the external $K^+$ concentration in the presence of DCCD. The Linweaver-Burk plots in the presence and absence of DCCD intersect near the vertical axis. Kinetic constants have been determined from plots such as are depicted in Figure 2. The apparent Km for $K^+$ increases from 7.2 $\pm$ 2.8 mM for control samples to 15.4 $\pm$ 5.0 mM in the presence of DCCD (average values from 10 experiments $\pm$ standard deviations). The measured Vmax of $K^+$ influx remains approximately constant at 3.3 $\pm$ 1.0 and 3.1 $\pm$ 1.1 $\mu$moles $K^+$ per gram of protein per minute in the absence and presence of DCCD.

The effect of pH on $K^+$ influx in the presence and absence of DCCD is shown in Figure 3. The rate of $K^+$ influx determined for control samples increases with increasing external $OH^-$ concentration, as in previous experi-



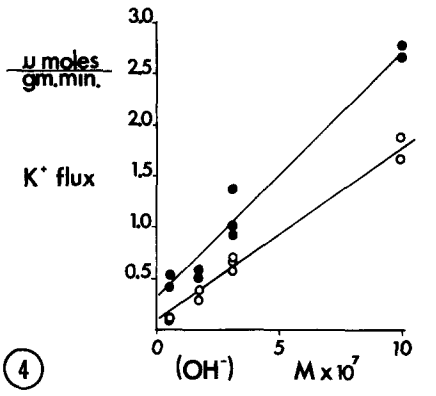




Figure 3: Dependence of $K^+$ influx on external $OH^-$ concentration in the presence and absence of DCCD. The mitochondrial protein concentration was 6.5 mg/ml. The pH of the medium, which contained 2.6 mM $K^+$, was varied from 6.8 to 8.0. The $K^+$ influx rate, in units of μmoles $K^+$ (gram protein)$^{-1}$ (min)$^{-1}$ is plotted against the molar $OH^-$ concentration in the medium. Symbols: ●, control samples; ○, the mitochondria were pretreated with DCCD (31 nmoles/mg protein).

Figure 4: Effect of DCCD on the dependence of $K^+$ influx on external $OH^-$ concentration in the presence of NEM. Conditions were the same as in Figure 3 except that the medium included 0.5 mM NEM. The $K^+$ influx rate, in units of μmoles $K^+$(gram protein)$^{-1}$(min)$^{-1}$ is plotted against the molar $OH^-$ concentration in the medium. The lines drawn are calculated by the method of least squares. Symbols: ●, control samples; ○, the mitochondria were pretreated with DCCD (31 nmoles/mg protein).

ments (10). DCCD inhibits $K^+$ influx at each of the pH values tested from 6.8 to 8.0. It has been consistently observed that DCCD decreases the stimulation of $K^+$ influx as the pH of the medium is raised.

The experiment shown in Figure 4 examines the effect of DCCD on the pH-dependence of $K^+$ influx in the presence of NEM, an inhibitor of the mitochondrial phosphate/hydroxyl exchange mechanism (20). The dependence of $K^+$ influx on external $OH^-$ concentration becomes linear in the presence of NEM, as reported previously (10). It has been suggested that this linear relationship may more accurately reflect the pH-dependence of the $K^+$ transport mechanism, since NEM prevents dissipation of experimentally manipulated pH gradients via exchanges of endogenous $P_i$ (10). The dependence of $K^+$ influx on external $OH^-$ concentration remains linear in the presence of DCCD. $K^+$ influx is slower in the presence of DCCD at each $OH^-$ concentration tested.

The calculated slopes of plots such as are depicted in Figure 4 have consistently shown a slight decrease in the pH-dependence of $K^+$ influx in the presence of DCCD.

## DISCUSSION

DCCD increases the apparent Km for $K^+$, while having little effect on the Vmax of $K^+$ influx. Since DCCD is a covalent inhibitor (1,2), these results cannot be interpreted as evidence for competition between $K^+$ and DCCD. The results rather indicate that DCCD decreases the affinity for $K^+$ of themitochondrial $K^+$ transport mechanism.

Beechey and coworkers have reported that following prolonged incubation of mitochondrial membranes with ($^{14}$C)DCCD at 4° C, 76% of the label was associated with a proteolipid fraction (1). Within this fraction, 90% of the label was associated with a protein later identified as the DCCD-binding subunit of the ATP synthase (1,2). Gel electrophoresis of beef heart submitochondrial particles treated with ($^{14}$C)DCCD has also shown the label to be associated with a single protein identifiable as the DCCD-binding ATP synthase subunit (21). Nevertheless, the possibility of multiple sites of action of DCCD cannot be ruled out. Thus DCCD may bind to a site on the $K^+$ transport mechanism which is separate from its site of binding on the ATP synthase.

The possibility may also be considered that DCCD may affect $K^+$ flux via its reaction with the ATP synthase. The differing effects of oligomycin and DCCD on $K^+$ flux are not necessarily inconsistent with this possibility. Effects of these chemically dissimilar reagents on the ATP synthase may differ. Furthermore, the lack of effect of oligomycin on $K^+$ influx is not inconsistent with the proposal (10) that $K^+$ influx may be directly coupled to $OH^-$ influx via the energy-linked transport mechanism. Oligomycin blocks "proton" fluxes in mitochondrial membranes stripped of the $F_1$ ATP-ase and in artificial membranes containing the isolated DCCD-binding proteolipid (3-5). The experiments showing no effect of oligomycin on $K^+$ influx were carried out with intact mitochondria. Some evidence indicates that the

isolated DCCD-binding protein can mediate oligomycin-sensitive $K^+$ as well as "$H^+$" fluxes in artificial lipid membranes (22).

Complex enzymes are known to exist in which binding of an inhibitor at one active site may alter kinetic characteristics at another site, for example aspartokinase I-homoserine dehydrogenase I of E. coli (23). Interpretation of the data in terms of a direct role of the ATP synthase in $K^+$ transport would require assuming that ion transport and oxidative phosphorylation are two functions of this enzyme complex which are not obligatorily linked, since DCCD affects only the apparent affinity of the transport mechanism for $K^+$ under conditions in which state 3 respiration is completely blocked. Recent observations of uncoupler-resistant mutants of Bacillus megaterium suggest that the coupling between ATP synthesis and ion transport may not be obligatory (24).

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