

Inhibition of the Mitochondrial Inner Membrane Anion Channel by Dicyclohexylcarbodiimide

EVIDENCE FOR A SPECIFIC TRANSPORT PATHWAY*

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Electrophoretic uniport of anions through the inner mitochondrial membrane can be activated by alkaline pH or by depleting the matrix of divalent cations. It has also been suggested that, in the presence of valinomycin and potassium, respiration can also activate anion uniport. We have proposed that a single pathway is responsible for all three of these transport processes (Garlid, K. D., and Beavis, A. D. (1986) *Biochim. Biophys. Acta* 853, 187-204). We now present evidence that like the "pH-dependent" pore the divalent cation-regulated pore and the "respiration-induced" pore are blocked by *N,N'*-dicyclohexylcarbodiimide (DCCD). Moreover, the kinetics of inhibition of the latter two pathways are identical and exhibit a second order rate constant of 2.6×10^{-3} (nmol DCCD/mg) $^{-1}$. min $^{-1}$. DCCD inhibits the uniport of Cl $^{-}$, phosphate, malate, and other lipophobic anions completely, but it has no effect on the classical electroneutral phosphate and dicarboxylate carriers. In Mg $^{2+}$ -depleted mitochondria DCCD partially inhibits the transport of SCN $^{-}$; however, in Mg $^{2+}$ -containing mitochondria and at low pH, no inhibition is observed. Furthermore, in DCCD-treated mitochondria, even following depletion of Mg $^{2+}$, the transport of SCN $^{-}$ is independent of pH. These results lead us to conclude that two pathways for anion uniport exist: a specific, regulated pathway which can conduct a wide variety of anions and a nonregulated pathway through the lipid bilayer which only conducts lipid-soluble ions.

Normal, freshly isolated mitochondria possess a very low electrophoretic permeability to most anions, with the exception of anions which are capable of delocalizing their electric charge such as SCN $^{-}$ and ClO $_{4}^{-}$. Twenty years ago, however, the groups of Azzone (1-4) and Brierley (5-7) demonstrated that at elevated pH the inner membranes of liver and heart mitochondria, respectively, become permeable to many different inorganic and organic anions. Recently, we presented evidence that an anion uniport pathway can be opened also at pH 7.4 by depleting the matrix of divalent cations (8). In addition, we demonstrated that this pathway can transport a

wide variety of anions and that it is also regulated by protons. These latter findings led us to propose that the pH- and Mg $^{2+}$ -regulated¹ pathways are identical (8, 9).

A question still unanswered is whether the change in permeability induced by Mg $^{2+}$ depletion and/or alkaline pH represents an opening of a specific pore or a change in the permeability properties of the lipid bilayer. In recent years, Selwyn's group (10) has demonstrated that at elevated pH electrophoretic transport of bicarbonate takes place in mitochondria but not in liposomes and has suggested that a specific "pH-dependent anion-conducting pore" exists in mitochondria (11). Furthermore, they have presented evidence that this pore is blocked by *N,N'*-dicyclohexylcarbodiimide (12).

DCCD is a hydrophobic alkylating agent which can react with a number of different groups on proteins and lipids including thiols and carboxyl groups. In the past few years it has proved to be a very useful tool in the study of a number of bioenergetically important proteins of the inner mitochondrial membrane including the F $_1$ F $_0$ -ATPase (13), complex III (14-16), complex IV (17), the transhydrogenase (18, 19), and the K $^{+}$ /H $^{+}$ antiporter (20-22). Thus, although the finding that DCCD inhibits anion uniport is consistent with the existence of a protein pore, by itself this finding does not rule out the possibility that transport occurs through the lipid bilayer. In fact, Jung *et al.* (23) have reported that DCCD blocks monovalent cation uniport through the inner membrane of heart mitochondria and have attributed this effect to blockage of a nonspecific leak.

We have now investigated the effect of DCCD on the divalent-cation-regulated anion uniport pathway and a number of other transport processes in mitochondria. The results indicate that DCCD completely blocks anion uniport through the putative pore but has no effect on uniport of SCN $^{-}$ through the lipid bilayer or electroneutral phosphate and dicarboxylate transport via the classical anion carriers. These findings provide further support for the proposal that the pH-dependent pore and the divalent cation-regulated pathway are identical and suggest that these transport processes do not reflect leakage through the lipid bilayer. Some of these data have been presented elsewhere in a preliminary report (24).

EXPERIMENTAL PROCEDURES

Rat liver mitochondria were prepared as previously described (25). Respiratory control was determined in a medium containing the K $^{+}$

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¹ The abbreviations used are: Mg $^{2+}$, divalent cation; EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazide; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; L.S., light scattering; mosm, milliosmolal; nosm, naniosmolal; DCCD, *N,N'*-dicyclohexylcarbodiimide.

salts of Cl (120 mM), TES (5 mM), succinate (5 mM), P_i (2 mM), and EGTA (0.5 mM) plus rotenone (1 $\mu\text{g}/\text{mg}$). Mitochondria were used at about 2 mg of protein/ml. Anion uniport was assayed by following swelling, which accompanies net salt transport, using the light scattering technique as described in detail elsewhere (25, 26). Using this technique we generate a light scattering variable, β , which normalizes reciprocal absorbance for mitochondrial protein concentration, P (milligrams/ml), according to the formula

$$\beta = \frac{P}{P_s} (A^{-1} - a)$$

where a is a machine constant and P_s (equals 1 mg/ml) is a constant introduced to make β dimensionless. Note that β increases as the mitochondrial matrix volume increases.

The rate of salt transport is calculated from the rate of change of β according to the formula (26)

$$J_s = \frac{\phi S_o}{nb} \left(\frac{d\beta}{dt} \right)$$

where ϕ is the medium osmolality (110 mosm in most studies reported here), S_o is the solute content of the stock preparation of mitochondria (190 nosm/mg), b (15 mosm) is the slope of the equilibrium absorbance osmotic curve (25), and n is the number of moles of osmotically active particles which make up 1 mol of the transported salt. At $\phi = 110$ mosm $\phi S_o/b$ is about 1400 nosm/mg.

For most experiments mitochondria were treated with DCCD (50 nmol/mg) in the stock suspension (50 mg/ml in 0.25 M sucrose) for a minimum of 45 min. DCCD was added as a 50 mM ethanolic solution, and controls were treated with an equal volume of ethanol for same length of time as the DCCD-treated mitochondria. To examine the kinetics of inhibition by DCCD, mitochondria were diluted to 10 mg/ml in a medium with the following final composition: K^+ salts of TES (65 mM), EGTA (0.5 mM), $MgCl_2$ (0.1 mM), and sucrose (50 mM from the mitochondria stock suspension). The temperature was maintained at 0 °C, and the pH was adjusted to 7.0 (at 25 °C). Samples were withdrawn at intervals and transferred to the assay medium which was maintained at 25 °C and pH 7.4. This medium contained the NH_4^+ salts of Cl (55 mM) or SCN^- (55 mM), TES (5 mM), EGTA (0.1 mM), and EDTA (0.1 mM). A23187 (10 nmol/mg), CCCP (10 nmol/mg), and rotenone (2 $\mu\text{g}/\text{mg}$) were added at zero time. In all L.S. assays the final concentration of mitochondria was close to 0.1 mg/ml.

RESULTS

DCCD Blocks Anion Uniport Induced by Depletion of Divalent Cations—Fig. 1 shows typical L.S. traces of control and DCCD-treated mitochondria suspended in a KCl assay medium. Addition of A23187 depletes the matrix of divalent cations and (in the presence of nigericin) induces a small increase in β which reflects the increase in volume associated with the net exchange of osmotically inactive matrix Mg^{2+} for osmotically active K^+ . Although in control mitochondria (trace a) depletion of matrix divalent cations activates the uniport pathway for Cl^- , no swelling is observed until valinomycin is added to provide a uniport pathway for K^+ (8). With DCCD-treated mitochondria, however, addition of A23187 and valinomycin does not induce swelling, indicating that Cl^- transport has been inhibited. Similar results were obtained with all other anions tested including malonate, citrate, sulfate, ferrocyanide, and nitrate (not shown). Inhibition of phosphate uniport by DCCD was demonstrated in a similar way using mitochondria treated with *N*-ethylmaleimide to block the electroneutral transport of phosphate by the phosphate $^-H^+$ symporter (not shown).

The Anion Uniport Pathway Is Independent of the Dicarboxylate Carrier and the Phosphate Carrier—In view of the finding that the anion uniport pathway carries phosphate, malonate, malate, citrate (8), and probably most other substrates for the dicarboxylate and tricarboxylate carriers, the question arises as to whether depletion of matrix Mg^{2+} or elevated pH could cause a conformational change in one or

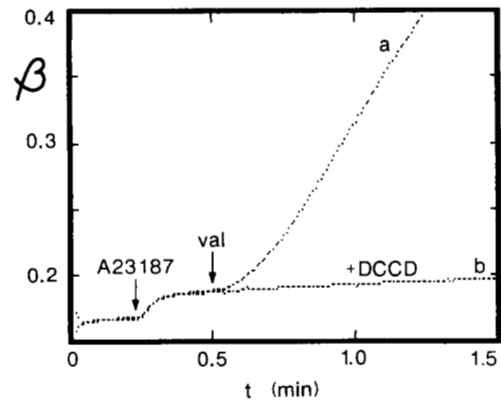


FIG. 1. DCCD inhibits the uniport of chloride. L.S. kinetics of mitochondria (0.1 mg/ml) suspended in KCl assay medium are shown. A23187 (10 nmol/mg) was added at 0.25 min and valinomycin (val, 0.5 nmol/mg) was added at 0.5 min. Trace a, control mitochondria. Trace b, mitochondria pretreated with DCCD (50 nmol/mg) for 45 min at 0 °C. The assay medium contained the K^+ salts of Cl^- (55 mM), TES (5 mM), EDTA (0.1 mM), and EGTA (0.1 mM) and was maintained at pH 7.4 and 25 °C. Rotenone (0.2 $\mu\text{g}/\text{mg}$) and nigericin (0.5 nmol/mg) were added at zero time. Rates of Cl^- transport calculated from these L.S. kinetics, as described under "Experimental Procedures," are 250 and 7 nmol of $Cl^-/\text{min}\cdot\text{mg}$ for traces a and b, respectively.

more of these carriers which might permit them to catalyze uniport. To examine this possibility, we determined the effect of DCCD on the activity of the phosphate, dicarboxylate, and tricarboxylate carriers using the classical swelling assays (27). Fig. 2A, shows typical results obtained with ammonium malate medium, in which swelling occurs upon the addition of a catalytic concentration of phosphate. This reflects the combined activities of the dicarboxylate carrier which exchanges medium malate $^{2-}$ for matrix P_i^{2-} and the phosphate carrier which catalyzes the net influx of phosphoric acid leading to net uptake of ammonium malate. Thus, if DCCD could block either of these pathways, phosphate-dependent swelling would be inhibited. Examination of Fig. 2A reveals no difference between the traces obtained with normal and DCCD-treated mitochondria. Similar results were obtained using ammonium citrate medium to assay the tricarboxylate carrier (not shown). To establish that the treatment with DCCD had indeed blocked the uniport pathway, we assayed for malate uniport using the same stock suspensions of mitochondria and the same ammonium malate assay medium. Fig. 2B shows that the DCCD-treated mitochondria do not swell following addition of the protonophore CCCP and A23187 (trace a), whereas the control mitochondria do (trace b). Thus, since DCCD appears to block malate uniport completely but has no effect on the electroneutral anion transporters, we conclude that the uniport pathway is independent of these carriers.

DCCD Blocks Chloride Transport Induced by Valinomycin in Respiring Mitochondria—Brierley (6) has reported that respiring heart mitochondria suspended in KCl swell rapidly upon the addition of valinomycin. This finding was confirmed in liver mitochondria of Azzone's group (28). Brierley (6) attributed the transport of Cl^- to the activation of a Cl^- uniport pathway. To determine whether this pathway could be the same as the pH-dependent pore (11) and the divalent cation-regulated pathway, we have examined the effect of DCCD on this process.

Comparison of traces a and b of Fig. 3 reveals that treatment of mitochondria with DCCD inhibits the swelling induced by the addition of valinomycin in respiring mitochondria by more than 95%. Although this result is consistent with inhibition

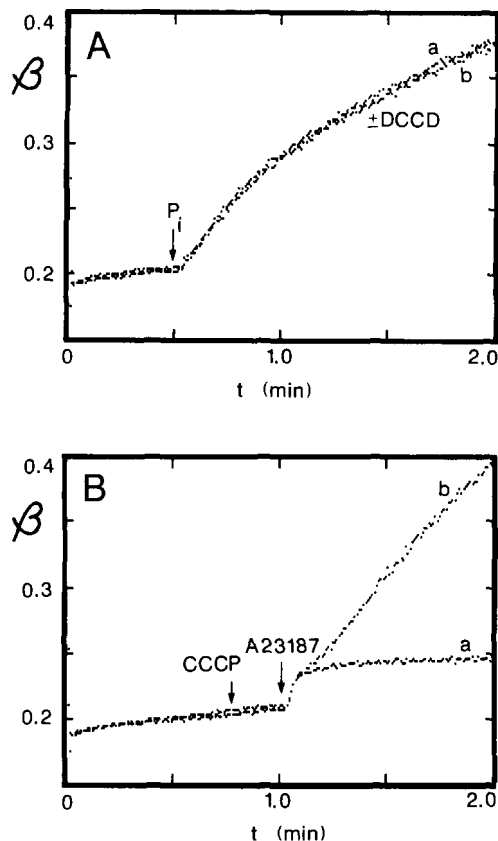


FIG. 2. DCCD blocks malate uniport but does not block the dicarboxylate or phosphate carriers. L.S. kinetics of mitochondria (0.1 mg/ml) suspended in ammonium malate assay medium are shown. A, phosphate (2.4 mM) was added at 0.5 min. Trace a, mitochondria pretreated with DCCD (50 nmol/mg) for 45 min at 0 °C; trace b, control mitochondria. B, CCCP (10 nmol/mg) was added at 0.8 min, and A23187 (10 nmol/mg) was added at 1.0 min. Trace a, mitochondria pretreated with DCCD as described for panel A; trace b, control mitochondria. The assay medium contained the ammonium salts of malate (37 mM), TES (5 mM), EDTA (0.1 mM), and EGTA (0.1 mM) plus rotenone (2 μ g/mg) and nigericin (0.5 nmol/mg) and was maintained at 25 °C and pH 7.4.

of Cl^- transport, it could also be explained by inhibition of electron flow or proton ejection. In fact, since DCCD is known to react with both complex III and complex IV of the respiratory chain (17), we carried out several experiments to investigate this possibility.

First, we determined whether DCCD-treated mitochondria were able to undergo respiration-driven swelling. To do this we utilized our finding that the classical phosphate and dicarboxylate carriers are not blocked by DCCD (Fig. 2). Thus, if the mitochondria were structurally intact and respiration was active, we would expect to observe swelling if we repeated the experiment shown in Fig. 3 in the presence of a low concentration of phosphate (2 mM) (29). With phosphate in the medium, the pH gradient generated by respiration upon the addition of valinomycin should drive the uptake of phosphate which in turn should exchange for succinate. Trace a of Fig. 4 demonstrates that under these conditions DCCD-treated mitochondria can swell at a rapid rate. This rate is about 10 times faster than the rate in the absence of phosphate (trace b of Fig. 3); however, it is also about 40% slower than the rate in non-DCCD-treated mitochondria (trace b). To exclude the possibility that phosphate could somehow activate Cl^- transport in DCCD-treated mitochondria, we carried out two further experiments.

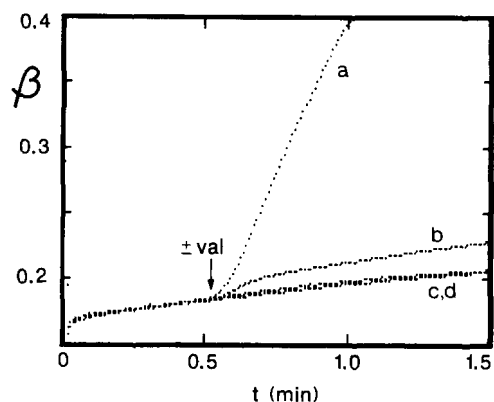


FIG. 3. The effect of DCCD on swelling induced by valinomycin in mitochondria respiring in the absence of permeant acids. L.S. kinetics of mitochondria (0.1 mg/ml) suspended in KCl assay medium are shown. Traces a and b, valinomycin (val, 0.5 nmol/mg) was added at 0.5 min to normal and DCCD-treated mitochondria, respectively. In both control (trace c) and DCCD-treated mitochondria (trace d), swelling is very slow in the absence of valinomycin. The assay medium contained the K^+ salts of Cl^- (55 mM), succinate (5 mM), TES (5 mM), and EGTA (0.1 mM) plus rotenone (2 μ g/mg) and was maintained at pH 7.4 and 25 °C. Mitochondria were pretreated with DCCD (45 nmol/mg) in the stock suspension for 30 min. Rates of Cl^- transport for traces a–d are 400, 33, 7, and 5 nmol Cl^- /min · mg, respectively, calculated from the L.S. kinetics as described under "Experimental Procedures."

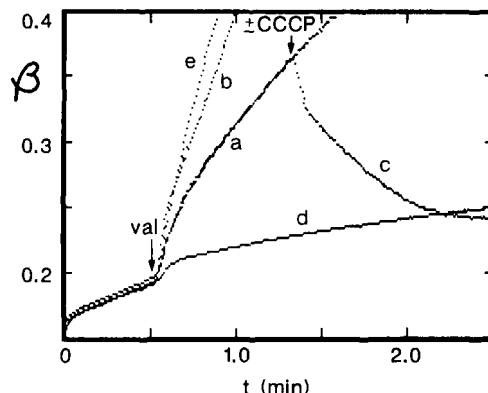


FIG. 4. The effect of DCCD on swelling induced by valinomycin in mitochondria respiring in the presence of permeant acids. L.S. kinetics of respiring mitochondria (0.1 mg/ml) suspended in KCl assay medium supplemented with succinate and phosphate are shown. Valinomycin (val, 0.5 nmol/mg) was added at 0.5 min. Trace a, mitochondria pretreated with DCCD (45 nmol/mg) taken from the same suspension as those used in Fig. 3; trace b, control mitochondria; trace c, same as trace a except CCCP (1 nmol/mg) was added where indicated; trace d, DCCD-treated mitochondria also treated with *N*-ethylmaleimide (40 nmol/mg); trace e, control mitochondria treated with *N*-ethylmaleimide. The assay medium was identical to that described in Fig. 3 except for the addition of potassium phosphate (2 mM).

First, on the basis of the following rationale we investigated whether the uncoupler CCCP would cause the swollen mitochondria to shrink. In the absence of phosphate, swelling is attributed to the passive influx of Cl^- down its concentration gradient and since the Cl^- gradient remains in the inward direction, no shrinkage is observed upon the addition of CCCP (not shown, but see Fig. 4 of Ref. 9). In contrast, uptake of phosphate and succinate via the electroneutral carriers is driven by the pH gradient. Consequently, since the concentration of these anions in the medium is relatively low, net uptake which is coupled to respiration will build up an outwardly directed anion gradient. Thus, addition of CCCP to

dissipate the pH gradient should cause shrinkage. Trace *c* of Fig. 4 confirms this prediction. In a second experiment we examined the effect of *N*-ethylmaleimide on the swelling in DCCD-treated mitochondria. Since this reagent inhibits the classical phosphate⁻H⁺ symporter it should block the swelling. This prediction is confirmed by trace *d* of Fig. 4. Note, that in non-DCCD-treated mitochondria, *N*-ethylmaleimide has no inhibitory effect on swelling (compare traces *b* and *e* of Fig. 4).

Since we wished to determine whether inhibition of electron flow could explain the inhibition of swelling observed in Figs. 3 and 4, in a second series of experiments we examined the effect of DCCD on respiration. In experiments parallel to those presented in Figs. 3 and 4, we found that DCCD inhibited respiration from a rate of 172 to 117 nmol O/min·mg (32% inhibition) and from 185 to 119 nmol O/min·mg (36% inhibition), respectively. We also determined the effect of DCCD on respiration rates in our normal respiration assay medium (see "Experimental Procedures"). State 4 respiration increased from 24.9 to 31.7 nmol O/min·mg; whereas the uncoupled rate (0.25 nmol CCCP/mg) was inhibited 44% from 207 to 116 nmol O/min·mg. Thus, although inhibition of respiration by DCCD is sufficient to explain the decrease in swelling rate in the presence of phosphate (Fig. 4), it cannot explain the inhibition in the absence of phosphate (Fig. 3).

Determination of the Rate Constant for Inhibition of Anion Uniport by DCCD—To examine the kinetics of inhibition of anion uniport by DCCD, mitochondria were treated with various doses of DCCD at 0 °C for a measured period and then transferred to the assay medium maintained at 25 °C (see "Experimental Procedures"). For these studies we assayed uniport of chloride in an NH₄Cl assay medium and added A23187 and CCCP at zero time but added no nigericin. These assay conditions minimize the lag between transfer of the mitochondria to the assay medium and the induction of maximal rates of anion uniport (8). This procedure was used to minimize the period of exposure of the mitochondria to DCCD at the elevated temperature of the assay and thus to minimize the extent of reaction which could take place during this period.

Typical results presented as semi-log plots are contained in Fig. 5A (open and closed circles). The linearity of these curves indicates that the rate of reaction is pseudo-first order. Fig. 5B shows the relationship between the first order rate constant k_1 and the concentration of DCCD. From this plot we have determined a value of 2.6×10^{-3} (nmol/mg)⁻¹·min⁻¹ for the second order rate constant. This constant is expressed in terms of nanomoles of DCCD/mg of protein since we have shown that under our treatment conditions essentially all the DCCD is bound to the mitochondria. A normal treatment mix was set up, and then the mitochondria were sedimented immediately after adding the DCCD by centrifuging the suspension in an Eppendorf centrifuge. Fresh mitochondria were then added to a sample of the supernatant and the time course of inhibition followed. Almost no inhibition could be detected when 50 nmol of DCCD/mg were used; however, some inhibition could be detected when 200 nmol of DCCD/mg were used. From pseudo-first order rate constant observed, we estimated that less than 2% of the DCCD remained in the supernatant. Note that controls were carried out to establish that the DCCD was not binding to the plastic Eppendorf tubes.

The qualitative finding that both M²⁺-regulated (Fig. 1) and "respiration-induced" (Fig. 2) swelling are inhibited by DCCD is consistent with, but does not prove, that they both reflect the activity of the anion uniport pathway. Conse-

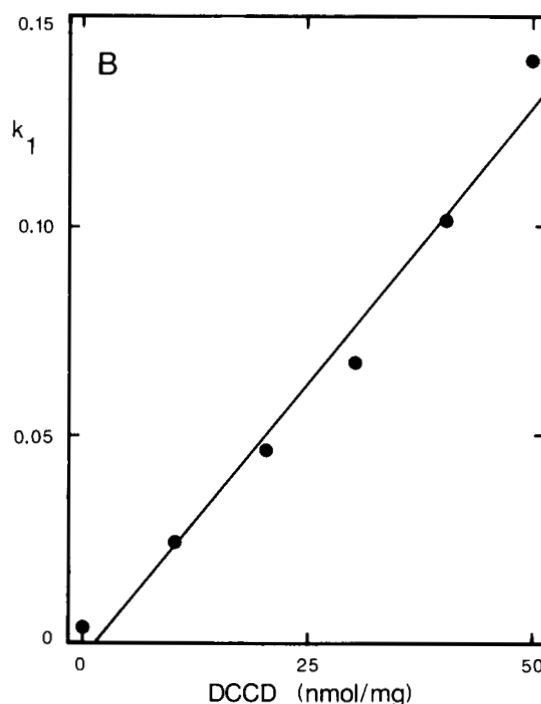
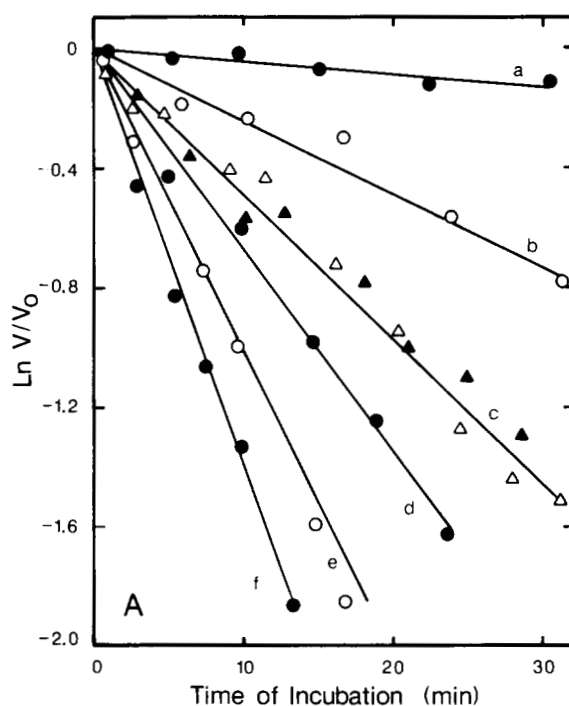


FIG. 5. Kinetics of inhibition of anion uniport by DCCD. A, $\ln V/V_0$ is plotted versus time of incubation with DCCD, where V is the rate of chloride uniport at the indicated time and V_0 is the rate at zero time. See "Experimental Procedures" for details of DCCD treatment and assay conditions. The curves correspond to DCCD concentrations (nanomoles/mg) of: a, 0; b, 10.5; c, 21; d, 30.5; e, 40.5; and f, 50. Curve *c* represents the results of an experiment in which swelling was assayed in both M²⁺-depleted mitochondria (\blacktriangle) as described under "Experimental Procedures" and in respiring mitochondria (\triangle) as described in the legend to Fig. 3. B, the pseudo-first order rate constants k_1 (min⁻¹) determined from the curves in A are plotted versus the amount of DCCD added to the incubation. The slope equals 2.63×10^{-3} (nmol/mg)⁻¹·min⁻¹.

quently, we also compared the kinetics of inhibition by DCCD using mitochondria from the same stock suspension. The result, also shown in Fig. 5A (*open and closed triangles*), reveals that the kinetics are indistinguishable and provides further evidence that these assays reflect the activity of the same transport pathway.

Inhibition of SCN^- Transport by DCCD Is Incomplete— SCN^- is a lipophilic anion which is thought to cross lipid bilayers (30, 31). However, in a previous communication (8) we showed that SCN^- transport is stimulated by depletion of matrix divalent cations and this led us to propose that this anion can be transported both via the lipid bilayer and the M^{2+} -regulated anion uniport pathway. The data contained in Fig. 6 show that, like endogenous divalent cations, DCCD only partially blocks the transport of SCN^- . Thus, while DCCD is able to block completely the transport of Cl^- in M^{2+} -depleted mitochondria (*closed circles*), under identical conditions it only blocks the transport of SCN^- by about 60% (*open circles*). Although these data are consistent with the existence of two pathways for the transport of SCN^- , they could also be explained by the existence of a single pathway through which the transport of SCN^- is only partially blocked by DCCD.

DCCD Has No Effect on M^{2+} - and H^+ -independent Transport of SCN^- —If SCN^- were transported via a single pathway, one might expect that DCCD would also partially inhibit SCN^- transport in normal M^{2+} -containing mitochondria. To investigate this we examined the effect of DCCD on swelling induced by nigericin plus CCCP in KSCN assay medium. Under these conditions, SCN^- transport is thought to occur

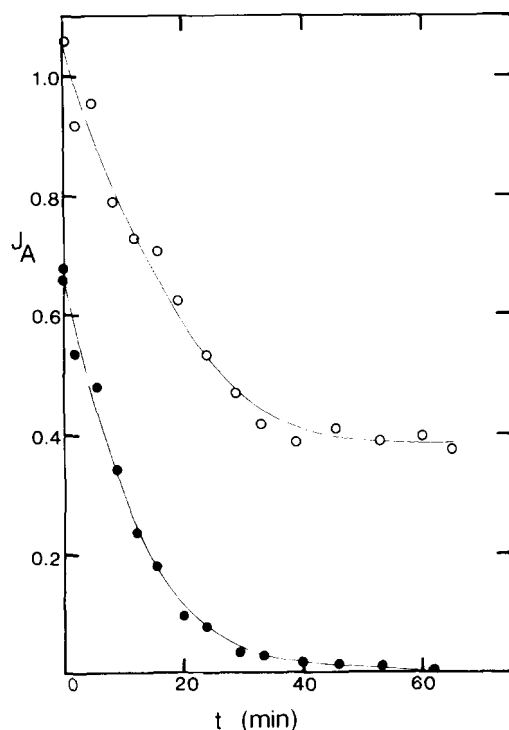


FIG. 6. The effect of DCCD on SCN^- and Cl^- transport in divalent cation-depleted mitochondria. The rate of anion transport (J_A , micromoles/min·mg) is plotted versus the duration of pretreatment of the mitochondria with DCCD (40 nmol/mg). The stock suspension of mitochondria was incubated with DCCD and samples were withdrawn at the times indicated and transferred to NH_4SCN (O) or NH_4Cl (●) assay medium. The maximum rate of anion transport was then calculated from the L.S. kinetics as described under "Experimental Procedures." The assay media are described under "Experimental Procedures."

solely via diffusion of the SCN^- anion through the lipid bilayer (9). Comparison of *traces a and b* in Fig. 7A reveals that DCCD has almost no effect on this process.

We have also determined the effect of DCCD on other mechanisms of SCN^- transport. The data contained in Fig. 7B demonstrate that DCCD also has essentially no effect on SCN^- transport induced by valinomycin in KSCN medium. Under these conditions, SCN^- transport is more rapid than that shown in Fig. 7A and this reflects the fact that SCN^- can be transported both electrophoretically as the anion and electroneutrally as a lipid-soluble ion pair with the valinomycin- K^+ complex (32, 33). The data in Fig. 7C show that DCCD also has no effect on SCN^- transport catalyzed by quinine. The protonated base of a hydrophobic drug, such as quinine, can form lipid-soluble ion pairs with SCN^- , and consequently the drug catalyzes net HSCN transport (9). As a result, in this assay, only the further addition of nigericin is necessary to permit net transport of KSCN.

In a fourth experiment we examined the effect of DCCD on SCN^- transport in M^{2+} -depleted mitochondria. To do this we carried out the experiment at pH 6.3 to keep the M^{2+} -regulated pathway closed (8). Under these conditions, SCN^- transport was rapid but unaffected by DCCD. Fig. 7D shows typical data obtained using valinomycin to induce net transport of KSCN.

Although in each of the four assays depicted in Fig. 7, A–D, the rate of swelling initiated by the addition of ionophore is unaffected by DCCD, it is evident that *traces a and b* in Fig. 7, A and B, tend to diverge as β rises above 0.3 (approximately 2-fold increase in matrix volume (25)). On the other hand, no divergence is observed in Fig. 7C (and Fig. 7D) even at β values up to 0.45. In view of the fact that quinine is able to inhibit the M^{2+} -regulated pathway (9, 24), this observation suggests that the divergence reflects swelling-induced activation of this transport pathway. Consistent with this conclusion is the additional finding that quinine eliminates the divergence observed in experiments of the type shown in Fig. 7, A and B (results not shown).

Permeability of the Lipid Bilayer Is Unaffected by pH—Like DCCD, protons also appear to block anion transport through the putative anion channel while having no effect on SCN^- permeation through the lipid bilayer in normal M^{2+} -containing mitochondria (8). Both results are consistent with the existence of two pathways for SCN^- transport; however, we could not be certain that the effect of pH did not reflect an increase in the permeability of the lipid bilayer which was only expressed following removal of membrane-bound M^{2+} . The finding that DCCD completely blocks the uniport of most anions has now allowed us to investigate the effect of pH on SCN^- transport in M^{2+} -depleted mitochondria with the putative anion uniporter closed. The result, shown in Fig. 8, clearly demonstrates that SCN^- transport in mitochondria which have been treated with DCCD and subsequently depleted of divalent cations is independent of medium pH from pH 6.6 to 8.4. Similar results were obtained using nigericin and CCCP to mediate net K^+ transport. These data, therefore, provide further evidence that the permeability of the lipid bilayer is unaffected by pH over the range found to modulate the M^{2+} -regulated anion uniport pathway.

DISCUSSION

In this paper we have presented evidence that DCCD inhibits M^{2+} -regulated anion uniport across the inner mitochondrial membrane without affecting a number of other transport processes. This result confirms our preliminary report (24) and provides further evidence that the pH-de-

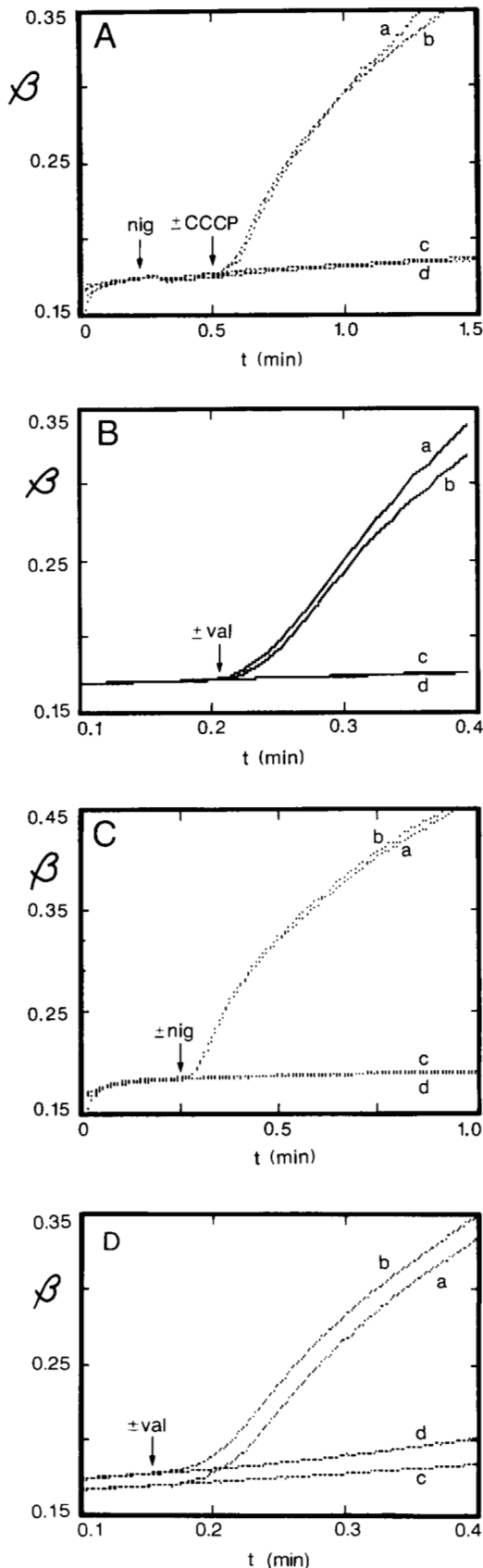


FIG. 7. The effect of DCCD on M^{2+} - and H^+ -independent SCN^- transport in nonrespiring mitochondria. L.S. kinetics of mitochondria (0.1 mg/ml) suspended in KSCN assay medium are shown. A, nigericin (*nig*, 1 nmol/mg) and CCCP (10 nmol/mg) were added where indicated to control (trace a) and DCCD-treated mitochondria (trace b). B, valinomycin (*val*, 0.5 nmol/mg) was added at

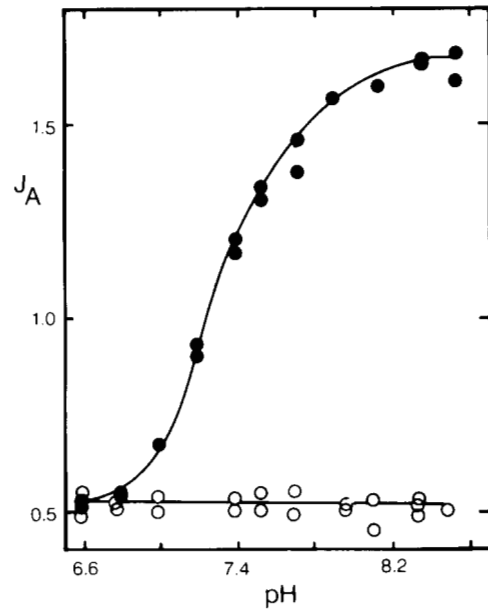


FIG. 8. The effect of DCCD on the pH dependence of SCN^- transport in divalent cation-depleted mitochondria. The rate of SCN^- transport (J_A , micromoles/min·mg) calculated from L.S. kinetics as described under "Experimental Procedures" is plotted versus the pH of the KSCN assay medium. Valinomycin (0.5 nmol/mg), nigericin (1 nmol/mg), A23187 (10 nmol/mg), and rotenone (2 μ g/mg) were included in the assay medium from zero time. ●, control mitochondria; ○, mitochondria pretreated with DCCD (50 nmol/mg) for at least 40 min. The assay medium contained the K^+ salts of SCN^- (55 mM), TES (5 mM), EDTA (0.1 mM), and EGTA (0.1 mM). The temperature was maintained at 25 °C and the pH adjusted with KOH.

pendent (11, 12) and the M^{2+} -regulated pathways are identical.

It has not yet been proved whether anion uniport induced by depletion of divalent cations or elevated pH is catalyzed by a protein or whether it represents a leak through the lipid bilayer. In view of the nonphysiological conditions necessary to observe rapid uniport, it occurred to us that the transport might be catalyzed by the classical electroneutral anion transporters. No evidence could be obtained, however, that DCCD affects the normal function of the phosphate, dicarboxylate, or tricarboxylate carriers in any way. This finding is contrary to the conclusion of Houstek *et al.* (34) who reported that DCCD partially blocks the phosphate carrier; however, it is consistent with the subsequent report by DePinto *et al.* (35) that DCCD does not bind to the phosphate carrier. In view of our findings, we suggest that the inhibition reported by Houstek *et al.* (34) reflects inhibition of phosphate uniport.

In addition to alkaline pH and depletion of divalent cations, the transport of Cl^- and other normally impermeant anions can be induced by the addition of valinomycin to respiring

0.5 min to control (trace a) and DCCD-treated mitochondria (trace b). C, quinine (0.5 mM) was included in the medium and nigericin (1 nmol/mg) was added at 0.25 min to control (trace a) and DCCD-treated mitochondria (trace b). D, valinomycin (0.5 nmol/mg) was added to control (trace a) and DCCD-treated mitochondria (trace b) suspended in KSCN medium containing A23187 (10 nmol/mg) and EDTA (0.1 mM) and adjusted to pH 6.3. In all four experiments, swelling rates were negligible in the absence of ionophores for both control (traces c) and DCCD-treated mitochondria (traces d). The assay medium contained the K^+ salts of SCN^- (55 mM), TES (5 mM), and EGTA (0.1 mM) plus rotenone (2 μ g/mg) and nigericin (1 nmol/mg) (when not added to initiate swelling) and was maintained at 25 °C. For A, B, and C, the pH was adjusted to pH 7.4. Mitochondria were pretreated with DCCD (45 nmol/mg) for at least 30 min.

mitochondria (Fig. 3). When Brierley (6) first observed this phenomenon he attributed it to the passive influx of K^+ via valinomycin coupled to the electrophoretic influx of Cl^- induced by the extreme alkalization of the matrix. We concur with this view and have proposed that the anion uniport observed under these conditions occurs via the same pathway which is opened up by depletion of divalent cations (9). Our finding that treatment of mitochondria with DCCD blocks this swelling and that this inhibition follows the same kinetics as inhibition of A23187-induced swelling provides strong evidence in support of this proposal.

The kinetics of inhibition of anion uniport by DCCD suggest that covalent modification of a single specific site is necessary to induce inhibition. The second order rate constant $2.6 \times 10^{-3} \text{ (nmol/mg)}^{-1} \cdot \text{min}^{-1}$ determined for our conditions of treatment is more than twice that reported for the K^+/H^+ antiporter (22). We should point out, however, that under the treatment conditions used here negligible inhibition of the K^+/H^+ antiporter takes place (22). This provides further evidence that these two pathways are physically independent (9). The question which remains to be answered is: At what site does DCCD react in order to inhibit anion uniport? Anion uniport is inhibited by protons, divalent cations, and DCCD; however we do not believe that all these reagents act at the same site. DCCD is highly hydrophobic, as evidenced by our finding that a negligible amount remains in the aqueous phase when it is added to a mitochondrial suspension. Thus, we believe that DCCD probably reacts at a site buried within the lipid phase of the membrane. On the other hand, divalent cations are hydrophilic and probably bind at a site accessible to the aqueous phase. In addition, we have no evidence that divalent cations are able to protect against inhibition by DCCD.

If anion uniport is not catalyzed by a membrane protein, but occurs through the lipid phase of the bilayer one might expect that the factors which modulate anion uniport would affect all transport through the lipid bilayer. Thus, we have sought factors which have a differential effect on the two pathways. Since lipid soluble anions such as SCN^- can cross the lipid bilayer (30, 31), we have investigated the effect of various agents on the transport of this anion. In this paper, we have shown that DCCD only partially blocks SCN^- anion transport in M^{2+} -depleted mitochondria (Fig. 6) and that it has virtually no effect in normal M^{2+} -containing mitochondria (Fig. 7A) and at low pH in M^{2+} -depleted mitochondria (Fig. 7D). Furthermore, electroneutral transport of SCN^- via ion-pair mechanisms (Fig. 7, B and C) also appears to be unaffected. Thus, DCCD appears to have no effect on the permeability of the lipid bilayer. This conclusion is consistent with our previous finding (26) that the transport of erythritol in mitochondria is also unaffected by DCCD. Thus, like divalent cations (8), DCCD can be used to distinguish two separate pathways for the transport of SCN^- .

In a previous communication (8), we showed that SCN^- transport in normal M^{2+} -containing mitochondria is unaffected by pH in the range from 6.6 to 8.4, whereas in M^{2+} -depleted mitochondria SCN^- transport is highly pH dependent. We cited this observation as evidence for the existence of two independent transport pathways for SCN^- , however the possibility remained that a single pathway was involved and that the permeability of the membrane only became pH

dependent upon removal of M^{2+} . We have now eliminated this possibility by showing that in DCCD-treated mitochondria the rate of SCN^- transport is independent of pH even in the absence of divalent cations (Fig. 8). If one accepts that SCN^- transport in DCCD-treated mitochondria occurs through the lipid bilayer, then it follows that pH has no effect on the permeability of the lipid bilayer, and, therefore, that pH-, M^{2+} -, and DCCD-dependent anion transport must occur via a completely independent pathway which is probably a protein.

In conclusion, the finding that DCCD irreversibly inhibits the putative inner membrane anion channel not only provides a powerful tool for further investigation of the properties of this transport pathway, but also provides a possible means of labeling and identifying the putative protein by gel electrophoresis.

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REFERENCES

- Azzi, A., Rossi, E., and Azzone, G. F. (1966) *Enzymol. Biol. Clin.* **7**, 25-37
- Azzi, A., and Azzone, G. F. (1966) *Biochim. Biophys. Acta* **120**, 466-468
- Azzi, A., and Azzone, G. F. (1967) *Biochim. Biophys. Acta* **131**, 468-478
- Azzone, G. F., and Piemonte, G. (1969) in *The Energy Level and Metabolic Control in Mitochondria* (Papa, S., Tager, J. M., Quagliariello, E., and Slater, E. C., eds) pp. 115-124, Adriatica Editrice, Bari
- Brierley, G. P. (1969) *Biochem. Biophys. Res. Commun.* **35**, 396-402
- Brierley, G. P. (1970) *Biochemistry* **9**, 697-707
- Brierley, G. P., and Stoner, C. D. (1970) *Biochemistry* **9**, 708-713
- Beavis, A. D., and Garlid, K. D. (1987) *J. Biol. Chem.* **262**, 15085-15093
- Garlid, K. D., and Beavis, A. D. (1986) *Biochim. Biophys. Acta* **853**, 187-204
- Selwyn, M. J., and Walker, H. A. (1977) *Biochem. J.* **166**, 137-139
- Selwyn, M. J., Dawson, A. P., and Fulton, D. V. (1979) *Biochem. Soc. Trans.* **7**, 216-219
- Warhurst, I. W., Dawson, A. P., and Selwyn, M. J. (1982) *FEBS Lett.* **149**, 249-252
- Beechey, R. B., Holloway, C. T., Knight, I. G., and Robertson, A. M. (1966) *Biochem. Biophys. Res. Commun.* **23**, 75-80
- Beattie, D. S., and Villalobo, A. (1982) *J. Biol. Chem.* **257**, 14745-14752
- Price, B. D., and Brand, M. D. (1983) *Eur. J. Biochem.* **132**, 595-601
- Degli Eposti, M., Meier, E. M. M., Timonedja, J., and Lenaz, G. (1983) *Biochim. Biophys. Acta* **725**, 349-360
- Azzi, A., Casey, R. P., and Nalecz, M. J. (1984) *Biochim. Biophys. Acta* **768**, 209-226
- Phelps, D. C., and Hatefi, Y. (1981) *J. Biol. Chem.* **256**, 8217-8221
- Pennington, R. M., and Fisher, R. R. (1981) *J. Biol. Chem.* **256**, 8963-8969
- Martin, W. H., Beavis, A. D., and Garlid, K. D. (1984) *J. Biol. Chem.* **258**, 2062-2065
- Garlid, K. D., DiResta, D. J., Beavis, A. D., and Martin, W. H. (1986) *J. Biol. Chem.* **261**, 1529-1535
- Martin, W. H., DiResta, D. J., and Garlid, K. D. (1986) *J. Biol. Chem.* **261**, 12300-12305
- Jung, D. W., Shi, G.-Y., and Brierley, G. P. (1980) *J. Biol. Chem.* **255**, 408-412
- Beavis, A. D., and Garlid, K. D. (1983) *Fed. Proc.* **42**, 1945
- Beavis, A. D., Brannan, R. D., and Garlid, K. D. (1985) *J. Biol. Chem.* **260**, 13424-13433
- Garlid, K. D., and Beavis, A. D. (1985) *J. Biol. Chem.* **260**, 13434-13441
- Chappell, J. B., and Haarhoff, K. N. (1967) in *Biochemistry of Mitochondria* (Slater, E. C., Kaniuga, Z., and Wojtczak, L., eds) pp. 75-91, Academic Press, New York
- Azzone, G. F., Massari, S., and Pozzan, T. (1976) *Biochim. Biophys. Acta* **423**, 27-41
- Chappell, J. B., and Crofts, A. R. (1966) in *Regulation of Metabolic Processes in Mitochondria* (Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., eds) Vol. 7, pp. 293-316, BBA Library, American Elsevier Publishing Co., New York
- Mitchell, P., and Moyle, J. (1969) *Eur. J. Biochem.* **9**, 149-155
- Lehninger, A. L. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 1520-1524
- Pressman, B. C. (1969) in *The Energy Level and Metabolic Control in Mitochondria* (Papa, S., Tager, J. M., Quagliariello, E., and Slater, E. C., eds) pp. 87-96, Adriatica Editrice, Bari
- DiResta, D. J., Beavis, A. D., and Garlid, K. D. (1985) *Biophys. J.* **47**, 486a
- Housteck, J., Pavelka, S., Kopecky, J., Drahota, Z., and Palmieri, F. (1981) *FEBS Lett.* **130**, 137-140
- DePinto, V., Tommasino, M., Bisaccia, F., and Palmieri, F. (1983) in *Structure and Function of Membrane Proteins* (Quagliariello, E., and Palmieri, F., eds) pp. 347-350, Elsevier Scientific Publishing Co., Inc., New York