

On the Mechanism by Which Dicyclohexylcarbodiimide and Quinine Inhibit K⁺ Transport in Rat Liver Mitochondria*

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Passive uptake of potassium acetate into the mitochondrial matrix can be induced by nigericin, a K⁺/H⁺ antiporter, or by A23187, a Mg²⁺/2H⁺ antiporter. The latter process is thought to reflect operation of the Mg²⁺-dependent, endogenous K⁺/H⁺ antiporter, but there is ambiguity with respect to the mechanism of K⁺ transport in this assay (Nakashima, R. A., and Garlid, K. D. (1982) *J. Biol. Chem.* 257, 9252-9254). Kinetic analysis of potassium acetate transport provides verification that Mg²⁺ depletion 1) unmasks the K⁺/H⁺ antiporter, 2) opens up an intrinsic anion uniporter, 3) has no effect on acetic acid transport, and 4) does not induce high K⁺ uniport conductance. Mg²⁺-dependent uptake of potassium acetate is thereby shown to be mediated specifically by operation of the endogenous K⁺/H⁺ antiporter, as previously proposed. An extension of this analysis confirms that N,N'-dicyclohexylcarbodiimide and quinine block potassium acetate uptake via specific action on the K⁺/H⁺ antiporter. These findings support those of a previous study (Martin, W. H., Beavis, A. D., and Garlid, K. D. (1984) *J. Biol. Chem.* 259, 2062-2065) in which binding of [¹⁴C]N,N'-dicyclohexylcarbodiimide to membrane proteins under selective conditions was used to identify an 82,000-dalton band as the protein responsible for K⁺/H⁺ antiport in mitochondria.

Chemiosmotic systems require an electroneutral mechanism for transport of alkali cations (1). In support of Mitchell's postulate (1), published 25 years ago, considerable evidence has been obtained for the existence of Na⁺/H⁺ and K⁺/H⁺ antiporters in the plasma membranes of prokaryotes and eukaryotes and in the inner membrane of mitochondria (for reviews, see Refs. 2-4). Since the protein responsible for K⁺/H⁺ antiport in mitochondria has been identified (5), the groundwork has been laid for isolation-reconstitution studies and for an investigation of the conservation, in nature, of this particular antiporter protein. The outcome of such a study depends strongly on the identification of an 82,000-dalton protein with K⁺/H⁺ antiport activity, and this identification, in turn, rests on the observation that DCCD¹ inhibits passive

swelling in KOAc medium (5). While it is likely that Mg²⁺-dependent uptake of KOAc into mitochondria is due to electroneutral transport of acetate (as HOAc) and K⁺ (via the K⁺/H⁺ antiporter), Mg²⁺ depletion may also open up uniport pathways for K⁺ and OAc⁻. Thus, the KOAc assay is ambiguous with respect to the underlying K⁺ transport pathway (6). If the mechanism of K⁺ transport is uncertain, then the mechanism of inhibition of this process is equally so. Further progress demands that this uncertainty be removed.

To this end, the present study addresses the following questions. 1) What intrinsic transport pathways are normally present in mitochondria for K⁺ and OAc⁻, and how are these affected by Mg²⁺ depletion? 2) Which transport pathways are being inhibited by DCCD and quinine in this assay? More specifically, do DCCD and quinine inhibit the endogenous K⁺/H⁺ antiporter? The experiments reported yield the gratifying results (a) that spontaneous swelling in KOAc can be shown to reflect K⁺/H⁺ antiport and not K⁺ uniport and (b) that DCCD and quinine each inhibit K⁺/H⁺ antiport under conditions favoring both influx and efflux of K⁺ via this pathway.

EXPERIMENTAL PROCEDURES

Mitochondrial Preparations—Rat liver mitochondria, isolated by differential centrifugation as previously described (7), were resuspended to 50 mg of protein/ml in 0.25 M sucrose and stored on ice. Mg²⁺-depleted mitochondria (5) were prepared by adding one part of stock suspension to four parts of 25 °C medium containing K⁺ salts of TES (27 mM) and EDTA (5.5 mM). The resulting mixture was 110 mosm and pH 7.8. Rotenone (1 nmol/mg) and A23187 (1 nmol/mg) were added, the suspension was incubated at 25 °C for 2 min to allow K⁺/H⁺ antiport to come to equilibrium, and then it was placed on ice. Mg²⁺ depletion after pretreatment was confirmed by atomic absorption spectroscopy (8). Control mitochondria, containing 35-40 nmol of endogenous Mg²⁺/mg (7), were treated exactly the same except that A23187 was omitted. For experiments using DCCD-treated preparations, control or Mg²⁺-depleted mitochondria were incubated with DCCD (50 nmol/mg) at 0 °C for 40 min prior to assay.

Ion Electrode Measurements—K⁺ fluxes in respiring mitochondria were measured as described by Dordick *et al.* (9). Data were collected by a Cyborg 91A analog/digital converter at 0.1-min intervals and passed to an Apple IIe computer for conversion and storage.

Light Scattering (L.S.) Measurements—Uptake of salts and water into the mitochondrial matrix results in matrix swelling and a consequent decrease in the light scattered by a mitochondrial suspension (10, 11). The L.S. variable β normalizes reciprocal absorbance (A^{-1}) for mitochondrial concentration, P (mg/ml):

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

where P_s (equals 1 mg/ml) is introduced to make β a scaled, dimensionless quantity, and a is a machine constant equal to 0.25 with our apparatus (7).

Absorbance was measured at 520 nm and sampled at 0.01-min intervals with a Brinkmann PC 700 probe colorimeter connected to a Cyborg 91A analog/digital converter. The digitalized signal was passed to an Apple IIe computer for conversion to inverse absorbance,

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¹ The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; TES, N-tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; L.S., light scattering; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid.

real time plotting, and storage. A linear regression routine was used to obtain initial rates, $v = d\beta/dt$ (min^{-1}), from the L.S. traces. Over the range studied, β and matrix water (W_m) are both linear with inverse osmolality (7):

$$\beta = \beta_0 + \frac{b}{\Phi} \quad (2)$$

$$W_m = W_b + \frac{S_0}{\Phi} \quad (3)$$

where Φ is medium osmolality, b (osmol) and S_0 (nosm/mg) are the observed slopes of the corresponding equilibrium osmotic curves, and β_0 and W_b (mg/mg) are the corresponding intercepts. W_m is matrix water content (sucrose-free water) determined from the distribution of [^{14}C]sucrose and $^3\text{H}_2\text{O}$ in parallel gravimetric experiments (7). Since water transport is much faster than salt transport (7), the rate of salt uptake, J (nmol/mg·min), is proportional to the L.S. kinetic, v (min^{-1}), and the proportionality constant is defined by Equations 2 and 3. For a 1:1 salt, ignoring the osmotic coefficient of the matrix solution,

$$J_{\text{salt}} = \frac{\Phi S_0}{2b} v. \quad (4)$$

S_0 is reproducibly found to be 190 nosmol/mg in our laboratory, and b is approximately 15 milliosmol with our equipment. At $\Phi = 110$ milliosmol, the proportionality constant is about 700 nmol/mg, and we can now relate transport kinetics to L.S. kinetics.

The 110 milliosmol assay medium for L.S. studies contains K^+ salts of acetate (55 mM; KOAc medium) or nitrate (55 mM; KNO_3 medium), TES (2 mM), EDTA (0.1 mM), and EGTA (0.1 mM), adjusted to pH 7.8.

RATIONALE²

RESULTS

Nigericin-induced Transport of KOAc—Fig. 2 contains Eadie-Hofstee plots of nigericin-induced swelling of normal and DCCD-treated mitochondria in KOAc, plotted according to Equation 27. The Eadie-Hofstee plots are linear, in accord with the kinetic model, and the results support both the validity of the underlying assumptions (see "Rationale") and the competence of the L.S. technique to yield quantitative transport kinetics. The v_{max} for the HOAc transport corresponds to 1800 nmol/mg·min, a rate that could not be measured by other means. Since HOAc is a very polar molecule, it seems likely that its high permeability is due to self-association as a shielded, hydrogen-bonded dimer. Both the v_{max} and the turnover number for the H^+ -nigericin/ K^+ -nigericin couple within the membrane are relatively unaffected by DCCD treatment, Mg^{2+} depletion, or quinine. The constancy of these kinetic parameters indicates that treatments used to activate or inhibit K^+/H^+ antiport do not induce a nonspecific increase in membrane permeability and specifically do not affect KOAc transport by modifying HOAc conductance through the membrane.

Pathway-specific Analysis of Mitochondrial Swelling in KOAc—Fig. 3 contains a series of representative traces from normal (Mg^{2+} -containing) mitochondria suspended in KOAc medium. The very low spontaneous swelling corresponds to 15 nmol/mg·min and confirms the absence of significant K^+/H^+ antiport activity in normal mitochondria (6). Addition of

² Portions of this paper (including "Rationale," Fig. 1, Table I, and Equations 5–28) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85 M-2613, cite the authors, and include a check or money order for \$4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

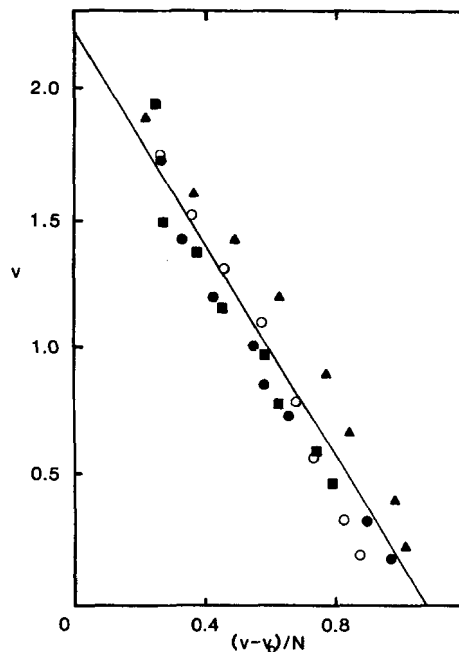


FIG. 2. Effect of Mg^{2+} depletion, quinine, and DCCD on nigericin-induced KOAc transport. Rates of mitochondrial swelling (v) were measured as described under "Experimental Procedures" and plotted versus $(v-v_0)/N$ according to Equation 27. Control mitochondria (●) were assayed directly for KOAc transport. Mg^{2+} -depleted mitochondria were assayed directly (■), in the presence of 0.15 mM quinine added to the assay medium (○), or after incubation with 50 nmol/mg DCCD at 0 °C for 40 min (▲). Swelling was initiated by addition of 100 μl of mitochondrial suspension to 10 ml of KOAc medium containing 0.1–10 nmol of nigericin (N). The endogenous rate (v_0) was obtained in the absence of nigericin.

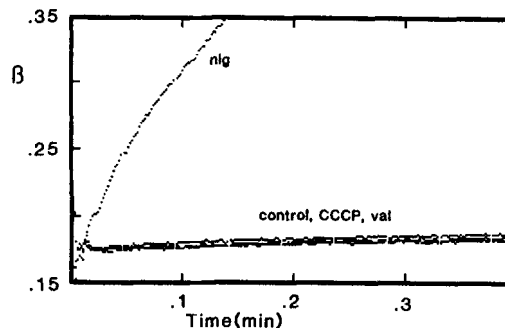


FIG. 3. Normal mitochondria are poorly permeable to K^+ , H^+ (OH^-), and anions. L.S. kinetics exhibited by control mitochondria in KOAc medium are shown. Where indicated, valinomycin (*val*) (0.2 nmol/mg), CCCP (10 nmol/mg), or nigericin (*nig*) (10 nmol/mg) was added to the assay medium immediately before the mitochondria.

CCCP or valinomycin does not affect the endogenous rate, demonstrating the low intrinsic conductance of the membrane to K^+ , H^+ , OAc^- , and OH^- (see "Rationale"). The high permeability of the membrane to acetic acid is reflected in the rapid swelling induced by nigericin.

Fig. 4 contains traces from mitochondria depleted of matrix Mg^{2+} . Spontaneous swelling is enhanced, corresponding to a K^+ transport rate of 200 nmol/mg·min. This rate is unaffected by CCCP, indicating that K^+ uniport does not contribute significantly to the spontaneous transport. The rate is stimulated by valinomycin, after a lag, in support of the contention (14) that Mg^{2+} depletion activates an endogenous uniport pathway for H^+ or OAc^- . The pattern exhibited in Fig. 4 is diagnostic for K^+ transport being mediated by K^+/H^+ antiport

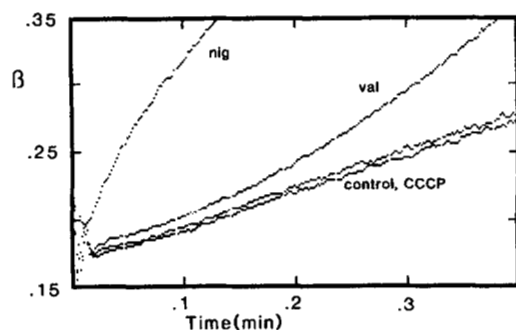


FIG. 4. Mg^{2+} depletion unmasks endogenous K^+/H^+ antiport activity. L.S. kinetics exhibited by Mg^{2+} -depleted mitochondria in KOAc medium are shown. When present, ionophores were added as described in the legend to Fig. 3.

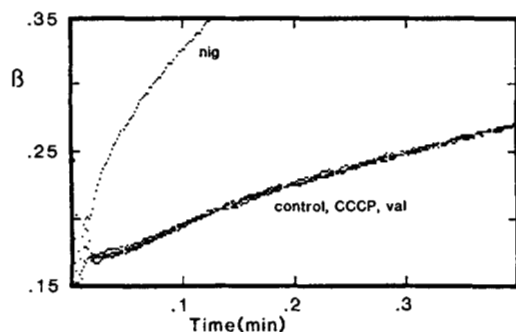


FIG. 5. Mg^{2+} protects K^+/H^+ antiport, but not anion uniport, from inhibition by DCCD. L.S. kinetics exhibited by Mg^{2+} -depleted mitochondria, treated with DCCD before Mg^{2+} depletion, in KOAc medium are shown. Stock mitochondria were incubated with 50 nmol/mg DCCD at 0 °C for 40 min, washed twice in isolation medium containing 0.2% bovine serum albumin, and resuspended at 50 mg/ml in 0.25 M sucrose. These mitochondria were subsequently depleted of Mg^{2+} and assayed.

and corresponds to Case C of Table I. We have carried out hundreds of similar experiments in Mg^{2+} -depleted mitochondria, and we invariably observe (a) a high rate of spontaneous swelling in KOAc which is inhibited by quinine (6) and, under appropriate conditions, by DCCD (5); (b) a considerable stimulation by valinomycin; and (c) a rate in the presence of CCCP which is 95–110% of the spontaneous rate. On the basis of the analysis presented under "Rationale," we conclude that K^+ uniport contributes no more than 15% of the total K^+ flux during KOAc uptake by Mg^{2+} -depleted mitochondria.

When mitochondria are treated with DCCD before Mg^{2+} depletion (see Fig. 5), stimulation of KOAc transport by valinomycin is selectively abolished, but spontaneous swelling is unaffected. This corresponds to Case A of Table I in which K^+/H^+ antiport is the only cation pathway available. The valinomycin-stimulated pathway is thereby shown to be independent of the K^+/H^+ antiport pathway, and its inhibition by DCCD supports its identification with the DCCD-sensitive anion uniporter (14, 15). K^+/H^+ antiport in DCCD-treated mitochondria (Fig. 5) is identical in its properties to that in non-DCCD-treated mitochondria (Fig. 4), in agreement with the conclusion of Martin *et al.* (5) that matrix Mg^{2+} protects the antiporter from attack by DCCD. In particular, the rates are nearly the same when care is taken to remove unreacted DCCD prior to Mg^{2+} depletion, using bovine serum albumin in the wash medium. The I_{50} for quinine inhibition is the same as that obtained with normal mitochondria (data not shown). The pseudo first-order rate constant for DCCD inhibition³ and the conditions for radiolabeling the 82,000-

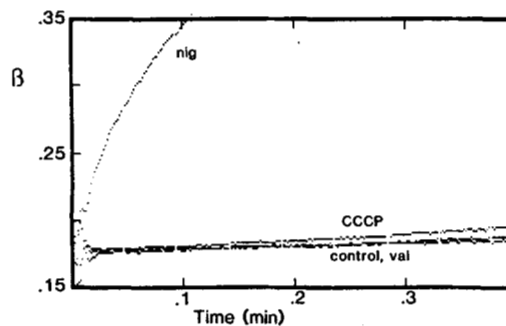


FIG. 6. DCCD inhibits K^+/H^+ antiport in Mg^{2+} -depleted mitochondria. L.S. kinetics exhibited by Mg^{2+} -depleted mitochondria, treated with DCCD before and after Mg^{2+} depletion, in KOAc medium are shown. Mitochondria were reacted with DCCD and depleted of Mg^{2+} as described for Fig. 5. Mg^{2+} -depleted mitochondria were then exposed again to 50 nmol/mg DCCD at 0 °C for 40 min and then assayed.

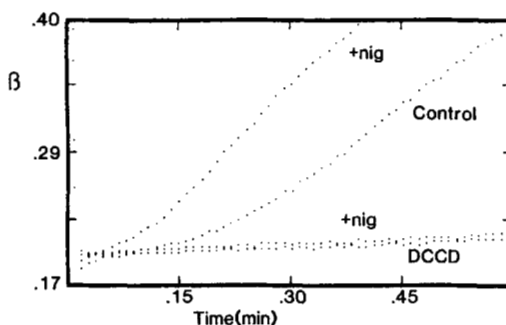


FIG. 7. Mg^{2+} does not protect KNO_3 transport from inhibition by DCCD. Swelling of Mg^{2+} -depleted mitochondria was assayed in KNO_3 medium containing 10 nmol/mg CCCP (control). When mitochondria were reacted with DCCD prior to Mg^{2+} depletion (see legend to Fig. 5), swelling was inhibited (DCCD). Including nigericin (10 nmol/mg) as well as CCCP in the assay medium stimulated the control rate of swelling but did not reverse the DCCD inhibition, showing that DCCD inhibits NO_3^- transport.

dalton band with [¹⁴C]DCCD are also unaffected by DCCD pretreatment under these conditions (5).

Fig. 6 contains traces from mitochondria which are first treated with DCCD, then depleted of Mg^{2+} prior to a second exposure to DCCD. The spontaneous swelling in KOAc is inhibited by 95% (compare Figs. 5 and 6), a dramatic illustration of the protective effect of Mg^{2+} against DCCD inhibition (5). Since K^+/H^+ antiport and HOAc uniport are the only pathways extant before and HOAc uniport is the only pathway extant after the second DCCD treatment, it follows that DCCD specifically inhibits K^+/H^+ antiport and that Mg^{2+} depletion is required to observe this effect (5).

DCCD Inhibition of KNO_3 Transport— KNO_3 transport in the presence of uncoupler is activated by Mg^{2+} depletion (Fig. 7), consistent with the unmasking of K^+/H^+ antiport and NO_3^- uniport pathways. DCCD inhibits swelling in KNO_3 , and inhibition does not require Mg^{2+} depletion prior to DCCD treatment (Fig. 7), in agreement with findings of Brierley *et al.* (16). The conclusion that endogenous Mg^{2+} does not always protect the K^+/H^+ antiporter against DCCD inhibition (16) represents a significant exception to previous findings (5); however, Brierley *et al.* (16) did not carry out the appropriate control experiment, which is to add both nigericin and CCCP to the assay in order to test for the effect of DCCD on NO_3^-

³ W. H. Martin and K. D. Garlid, unpublished data.

uniport. When this is done, DCCD is again found to inhibit swelling by 95% (Fig. 7), identifying the transport defect as inhibition of NO_3^- transport. Thus, we conclude that the finding of Brierley *et al.* (16) reflects inhibition of NO_3^- uniport by DCCD (an effect known not to require pre-depletion of Mg^{2+} (14, 15)) and has no bearing on Mg^{2+} protection of the K^+/H^+ antiporter.

Inhibition of the K^+/H^+ Antiporter by Quinine and Quinacrine—Fig. 8 contains dose-response curves for inhibition of swelling in KOAc by quinine and quinacrine. The curves correspond to a model with a Hill slope of 1.0 and an I_{50} for inhibition of $6 \mu M$ for quinacrine and $27 \mu M$ for quinine. The same values are obtained in mitochondria pretreated with DCCD under conditions of Fig. 5 (data not shown). Neither drug affects HOAc transport significantly (see, for example, Fig. 2), and we conclude that the site of drug action on K^+ transport is the K^+/H^+ antiporter, in agreement with previous conclusions (6).

DCCD Inhibition of K^+ Efflux from Respiring Mitochondria—The divalent cation ionophore A23187 induces K^+ efflux from respiring mitochondria (17, 18), and this has been shown to reflect unmasking of the endogenous K^+/H^+ antiporter secondary to divalent cation depletion (9). A23187-induced K^+ efflux and its reversal by valinomycin are seen in the data of Fig. 9. Since valinomycin catalyzes electrophoretic transport of K^+ , the gradient for K^+ uniport must be inward, and the loss of K^+ under these conditions must therefore occur via an endogenous, electroneutral K^+ transport pathway and not via a K^+ uniport pathway (9, 19–22). A23187-induced K^+ efflux from respiring mitochondria is thus a well-characterized process which provides an independent experimental model for evaluating the contention (5) that DCCD specifically inhibits the K^+/H^+ antiporter of mitochondria.

Pretreatment of normal, Mg^{2+} -containing mitochondria with DCCD does not inhibit K^+ efflux (data not shown),

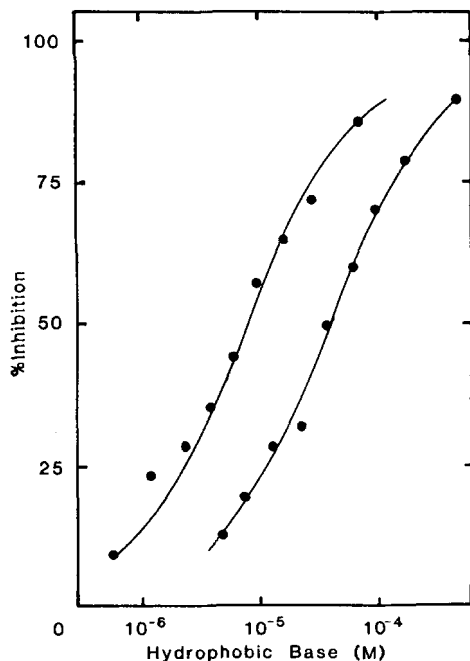


FIG. 8. Inhibition of potassium acetate transport by quinine and quinacrine. Mg -depleted mitochondria were assayed in KOAc medium containing quinacrine (left curve) or quinine (right curve) at doses indicated on the abscissa. The per cent inhibition of the control rate is plotted versus log of the drug concentration. The solid curves were drawn assuming a Hill slope of 1 and an I_{50} of $6 \mu M$ for quinacrine and $27 \mu M$ for quinine.

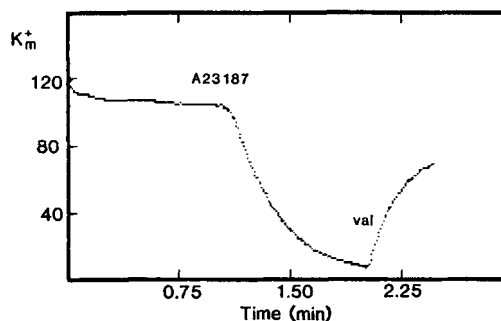


FIG. 9. Mg^{2+} -depletion activates K^+ efflux from respiring mitochondria. Matrix K^+ (K_m , nmol/mg), determined from ion electrode readings, is plotted versus time. The medium contains sucrose (38 mM), KCl (0.2 mM), rotenone (1 $\mu g/mg$), and trimethylamine salts of succinate (5 mM), phosphate (2 mM), EDTA (0.5 mM), EGTA (0.5 mM), Cl^- (20 mM), and TES (5 mM), pH 7.1. A23187 (1 nmol/mg) was added at 1 min, and valinomycin (0.5 nmol/mg) was added after 2 min.

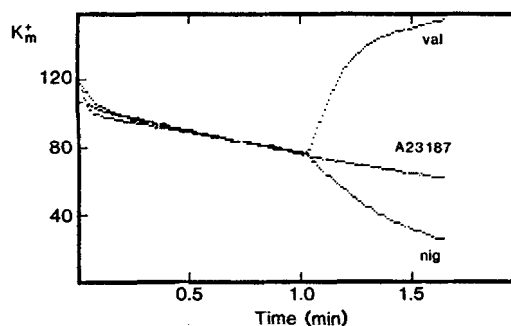


FIG. 10. DCCD inhibits K^+/H^+ antiport in respiring mitochondria. Matrix K^+ (K_m , nmol/mg), determined from ion electrode readings, is plotted versus time. Mg -depleted mitochondria were incubated with 50 nmol/mg DCCD at $0^\circ C$ for 40 min and washed twice in isolation medium containing 0.2% bovine serum albumin. Treated mitochondria were assayed in the same medium as described in the legend to Fig. 9. A23187 (1 nmol/mg), valinomycin (0.5 nmol/mg), or nigericin (3 pmol/mg) was added at 1 min.

consistent with the hypothesis that endogenous Mg^{2+} protects the antiporter from DCCD inhibition (5). When mitochondria are reacted with DCCD following depletion of matrix Mg^{2+} , the rapid K^+ loss observed in Fig. 9 is inhibited by 90% (Fig. 10). Addition of A23187 has no effect, which is not surprising in view of the fact that these mitochondria have already been treated with this ionophore to remove Mg^{2+} . Inhibition of K^+ efflux is not due to the effect of DCCD on respiration, since these mitochondria still exhibit a pronounced K^+ uptake following addition of valinomycin (Fig. 10). That these mitochondria remain coupled is further confirmed by the fact that CCCP is found to stimulate respiration, giving a respiratory control ratio of 3.8. In addition, these mitochondria have retained nearly all their matrix K^+ (110 nmol/mg) despite having been washed twice in sucrose after Mg^{2+} depletion. Non-DCCD-treated mitochondria, identically subjected to Mg^{2+} depletion and two washes, retain only 20–25 nmol/mg K^+ . Since the gradients for K^+/H^+ antiport and K^+ uniport, as determined by the responses to nigericin and valinomycin, are identical in the experiments of Figs. 9 and 10, we conclude that DCCD inhibits K^+ efflux by inhibiting the endogenous K^+/H^+ antiporter.

DISCUSSION

A physiological consequence of the chemiosmotic theory (1) is that mitochondria are subjected to a constant osmotic stress

in vivo, secondary to passive uptake of K⁺ salts and water. The electrochemical gradient favoring K⁺ uptake into the matrix is so large that mitochondria can be viewed as infinite sinks for K⁺. To compensate for electrophoretic cation influx, the inner membrane was postulated to contain cation/proton antiport for the electroneutral efflux of cations (1). The existence of an active Na⁺/H⁺ antiporter was demonstrated in mitochondria by Mitchell and Moyle (12), but K⁺/H⁺ antiport was exceedingly slow. Specifically, mitochondria swell only slowly in potassium acetate despite their high permeability to acetic acid (12, 13). The well-established observation that freshly isolated mitochondria retain K⁺ in isotonic sucrose medium (23) also appeared to argue against the existence of an intrinsic K⁺/H⁺ antiporter, since the gradient in such preparations strongly favors K⁺ efflux.

The apparent contradiction between experiment and physiological necessity was resolved by the demonstration (19) that moderate hypotonic swelling under mild conditions activates passive, electroneutral K⁺ efflux from mitochondria. Garlid (19–21) argued that volume homeostasis *in vivo* requires regulation of K⁺/H⁺ antiport and presented evidence for a primary role of Mg²⁺ ions, acting asymmetrically on the matrix side of the antiporter as a “carrier brake.” According to this model, retention of K⁺ in low-potassium media and failure to swell in potassium acetate medium both reflect inhibition of the K⁺/H⁺ antiporter by its endogenous regulator and not its absence.

The physiological role of the Mg²⁺-regulated K⁺/H⁺ antiporter was deduced from experiments on mitochondria containing their normal complement of Mg²⁺, about 38 nmol/mg. To discover other inhibitors of the antiporter and to learn more about the kinetics of K⁺/H⁺ antiport, it is necessary to remove the inhibitory influence of Mg²⁺. To compare transport of K⁺ with that of Na⁺ and other cations, it is necessary to devise conditions in which the gradients are comparable. Accordingly, our next approach was to seek conditions of Mg²⁺ depletion in which the gradient of K⁺/H⁺ antiport is inward and can be coupled to acetic acid uptake.

This approach proved successful, enabling us to show for the first time 1) that the K⁺/H⁺ antiporter is capable of bidirectional K⁺ transport (6); 2) that the K⁺/H⁺ antiporter is inhibited reversibly by quinine (6) and irreversibly by DCCD (5); 3) that mitochondria possess two different antiporters for alkali cations with different ion selectivities and inhibitor sensitivities (5, 6); and 4) that the K⁺/H⁺ antiporter is composed, entirely or in part, of an 82,000-dalton inner membrane peptide (5). These conclusions have far-reaching consequences for our understanding of this vital process. The caveat is that they rest on the assumption that uptake of KOAc occurs via K⁺/H⁺ antiport and not by K⁺ uniport.

It is difficult to distinguish electroneutral from electrophoretic K⁺ transport in nonrespiring mitochondria because the gradients are generally in the same direction and both processes involve coupled K⁺/H⁺ exchange (see model in Fig. 1). Nevertheless, we are able to exploit one meaningful distinction between these two modes of K⁺ transport. K⁺/H⁺ exchange on the antiporter is obligatorily coupled by the transport protein, and addition of an ionophore for K⁺ or H⁺ cannot affect this coupling. In contrast, K⁺/H⁺ exchange by uniport pathways is coupled *indirectly* by electrical forces, and addition of an ionophore for one of the ions will profoundly affect transport of the other.⁴ These physiocochemical criteria provide a powerful yet simple means to distinguish

between alternative ion transport pathways in nonrespiring mitochondria, as summarized in Table I and discussed under “Rationale.”

This approach has been subjected to an extensive series of studies, summarized in Figs. 2–6, on four different populations of mitochondria. Applying the criteria developed under “Rationale” (Table I) to these results, we conclude that Mg²⁺-dependent swelling in KOAc is mediated by K⁺/H⁺ antiport. Thus, depletion of matrix Mg²⁺ unmasks the endogenous K⁺/H⁺ antiporter (9, 21), and this antiporter, which is asymmetric with respect to Mg²⁺ inhibition (19, 21), is shown to be capable of bidirectional K⁺ transport (6). Mg²⁺ depletion also unmasks a uniport pathway for H⁺, OH⁻, or OAc⁻ (14). Mg²⁺ depletion appears not to induce significant K⁺ uniport activity, nor is the high endogenous permeability to HOAc affected. In studies published elsewhere (24), we have also found that Mg²⁺ depletion does not affect the permeability of the inner membrane to erythritol or to the quinine-thiocyanate ion pair (25). Thus, the unsupported statement that divalent cation depletion produces generalized increases in the permeability of the mitochondrial membrane (16) is found to be incorrect.

Failure to detect significant K⁺ uniport in these assays should not be taken to imply that such a pathway is absent. Using Equations 9 and 10 and assuming zero concentration gradient for K⁺, we can calculate the conductance ratio which must obtain *in vivo* during the steady state ($J_{K^+} + J_{K/H} = 0$):

$$\frac{L_{K^+}}{L_{K/H}} = \frac{59\Delta pH}{\Delta\psi}$$

Thus, L_{K^+} should be about 10% of $L_{K/H}$ *in vivo*. *In vitro*, we observe a lower ratio, about 1–1.5%, a reduction which can be attributed simply to stimulation of $L_{K/H}$ by Mg²⁺ depletion. The implication that the K⁺/H⁺ antiporter operates *in vivo* at 10–15% of its maximum rate is consistent with the proposal that this is an optimal level of regulation (8).

The present study validates the KOAc swelling assay (5, 6, 12) as an experimental model for Mg²⁺-dependent K⁺/H⁺ antiport in mitochondria. Nevertheless, it is reassuring to note that conclusions derived from this assay also apply to K⁺ efflux on the K⁺/H⁺ antiporter, where K⁺ transport can be measured directly with the ion electrode. Thus, DCCD, like quinine (6), inhibits electroneutral K⁺ efflux from respiring mitochondria (Fig. 10). The fact that endogenous K⁺ is not retained during sucrose washes by Mg²⁺-depleted mitochondria *unless* they are treated with DCCD further supports this mechanism of action. Valinomycin stimulates K⁺ uptake to higher than control levels, perhaps as a consequence of K⁺/H⁺ antiport inhibition and reduced futile cycling of K⁺.

Both quinine and quinacrine inhibit KOAc transport (Fig. 8). From similar results in beef heart mitochondria, Jung *et al.* (26) have questioned whether this effect is due entirely to inhibition of K⁺/H⁺ antiport and suggest that these drugs may act by inhibiting K⁺ uniport. We have shown 1) that K⁺ uniport does not contribute significantly to KOAc transport in Mg²⁺-depleted mitochondria (Fig. 4) and 2) that the Hill coefficient for inhibition of KOAc transport by these drugs is 1 (Fig. 8), consistent with inhibition of a single transport process. While these drugs are capable of affecting other mitochondrial ion transport processes (6, 25, 27), we conclude that they are inhibiting K⁺/H⁺ antiport specifically under these experimental conditions.

Brierley *et al.* (16) find that DCCD does not inhibit ⁴²K⁺/K⁺ exchange in beef heart mitochondria, a result which conflicts with findings of Gauthier and Diwan (28). Brierley *et al.* (16) conclude that either DCCD does not inhibit K⁺/H⁺ antiport or ⁴²K⁺/K⁺ exchange does not take place on the K⁺/

⁴ This statement also applies to electrically coupled uniports of K⁺ and OAc⁻. Because of the high permeability to HOAc (Fig. 2), inward OAc⁻ uniport is equivalent to outward H⁺ uniport (see “Rationale”).

H⁺ antiporter. The first alternative can be excluded by the present results. The second alternative, that ⁴²K⁺/K⁺ exchange is occurring via K⁺ uniport pathways, cannot be ruled out, since such protocols are unable to distinguish between uniport and antiport mechanisms and Brierley *et al.* (16) have not established that their beef heart preparations retain the low K⁺ uniport activity which we routinely observe in rat liver mitochondria (see Fig. 3). We offer an alternative possibility for the failure of DCCD to inhibit ⁴²K⁺/K⁺ exchange in beef heart mitochondria, namely, that DCCD selectively blocks proton transport on the K⁺/H⁺ antiporter without affecting K⁺/K⁺ exchange on the antiporter. Solioz (29) has suggested that DCCD may be selective for proton-translocating proteins, although this proposal has been criticized (30). It may be relevant in this regard that protons, but not potassium ions, protect the antiporter from DCCD inhibition and binding (5).

Brierley and co-workers (16, 26) have challenged our interpretation of KOAc uptake studies on the grounds that definitive evidence for participation of an endogenous K⁺/H⁺ antiporter is lacking in such assays. We said much the same thing in 1982 but argued on the basis of qualitative experiments that K⁺/H⁺ antiport was the likely mechanism (6). To obtain "definitive" evidence is a tall order, requiring both a theoretically sound, quantifiable model and a competent methodology. We have taken some pains to lay this groundwork (see Refs. 7 and 24 and "Rationale"), enabling us to evaluate the alternative hypothesis (16, 26) that passive KOAc uptake into mitochondria is mediated largely by K⁺ uniport. The present studies show this hypothesis to be false, thereby removing, in a Popperian sense, the ambiguity in the mechanism of K⁺ transport during passive KOAc uptake.

For the present, therefore, we conclude 1) that swelling in KOAc is mediated by K⁺/H⁺ antiport and not by K⁺ uniport, and 2) that quinine and DCCD inhibit Mg²⁺-dependent swelling by virtue of a specific action on the K⁺/H⁺ antiporter.

The evidence favoring these conclusions is sufficiently compelling to persuade us to continue in our efforts to characterize the 82,000-dalton protein which we have identified as the K⁺/H⁺ antiporter (5).

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Supplementary Material to

On the Mechanism by which Dicyclohexylcarbodiimide and Quinine Inhibit K⁺ Transport in Rat Liver Mitochondria

by Keith D. Garlid, Daniel J. DiResta, Andrew D. Beavis and William H. Martin

RATIONALE

Net KOAc transport across the inner membrane must be electrically neutral, a requirement which can be met by a variety of endogenous transport pathways, as illustrated in Fig. 1. The experimental problem is to identify the extent to which these pathways contribute to KOAc uptake. We begin with the overall transport equation for the flux of potassium acetate:

$$J_{\text{KOAc}} = -L_{\text{KOAc}} \nabla \mu_{\text{KOAc}} \quad (5)$$

Since water transport is orders of magnitude faster than salt transport, Equation 5 is a complete phenomenological description of KOAc transport. We next introduce the extrathermodynamic and testable assumption that ionophore-mediated transport pathways are ion-specific and independent of the endogenous pathways. The general approach is to determine how L_{KOAc} depends on the individual conductances of the 5 intrinsic, ion-specific pathways portrayed in Fig. 1. In each case, criteria are developed to determine experimentally whether the putative pathways exist or not. Thus, the following analysis is impartial with respect to the mechanism of K⁺ transport.

The Transport Equations - The combination of OAc⁻ uniport with HOAc uniport will be indistinguishable from H⁺ uniport if HOAc conductance is very high, as is commonly assumed (12,13). Accordingly, OAc⁻ and H⁺ (or OH⁻) uniport can be lumped together in J_{H^+} for present purposes, and the number of pathways is reduced from five to four. The remaining pathway-specific fluxes are related to the total transport by the following electroneutrality requirements:

$$J_{\text{KOAc}} = J_{\text{HOAc}} = J_{\text{K}^+} + J_{\text{K}/\text{H}} \quad (6)$$

$$J_{\text{H}^+} = -J_{\text{K}^+} \quad (7)$$

The force-flux relationships can be written:

$$J_{\text{HOAc}} = -L_{\text{HOAc}} \nabla \mu_{\text{HOAc}} \quad (8)$$

$$J_{\text{K}/\text{H}} = -L_{\text{K}/\text{H}} (\nabla \mu_{\text{K}^+} - \nabla \mu_{\text{H}^+}) \quad (9)$$

$$J_{\text{K}^+} = -L_{\text{K}^+} \nabla \mu_{\text{K}^+} \quad (10)$$

$$J_{\text{H}^+} = -L_{\text{H}^+} \nabla \mu_{\text{H}^+} \quad (11)$$

To aid in solving Equations 5-11, we introduce a parameter, f , defined as the fraction of total flux contributed by K⁺ uniport:

$$f = J_{\text{K}^+} / J_{\text{KOAc}} \quad (12)$$

1/f is found to be given by

$$\frac{1}{f} = 1 + \frac{L_{\text{K}/\text{H}} (L_{\text{K}^+} + L_{\text{H}^+})}{L_{\text{K}^+} L_{\text{H}^+}} \quad (13)$$

Using Equation 4, the general solution for J_{KOAc} can be expressed in terms of observed swelling rate, v :

$$\frac{1}{v} = \frac{1}{L_{\text{HOAc}}} + \frac{(1-f)}{L_{\text{K}/\text{H}}} \quad (14)$$

Where, for simplicity, we have lumped constants and introduced relative conductances, L'_{K^+} , defined by

$$L'_{\text{K}^+} = \frac{2b \nabla \mu_{\text{KOAc}}}{S_0 \Phi} L_{\text{K}^+} \quad (15)$$

We assume that a given ionophore makes the appropriate conductance infinitely large with respect to all other conductances. Consequently, Equation 14 yields four independent expressions corresponding to the rates measured in the absence of ionophores (v₀) and in the presence of nigericin (v_N), valinomycin (v_V) or CCCP (v_C). The solutions for f and the four relative conductances are given in Equations 16-20:

$$f^2 = \frac{(v_C/v_0 - 1)(v_V/v_0 - 1)}{(1 - v_C/v_N)(1 - v_V/v_N)} \quad (16)$$

$$L'_{HOAc} = v_N \quad (17)$$

$$L'_{K/H} = \frac{v_0 v_N}{v_N - v_0} (1 - f) \quad (18)$$

$$L'_{K^+} = \frac{v_N v_C}{v_N - v_C} - L'_{K/H} \quad (19)$$

$$L'_{H^+} = \frac{v_N v_V}{v_N - v_V} - L'_{K/H} \quad (20)$$

Acetic Acid Permeability - The relative permeability of the inner membrane to HOAc can be estimated with the aid of nigericin, which catalyzes K⁺/H⁺ antiport. In this case, it is sufficient to consider the combination of K⁺/H⁺ antiport and HOAc uniport pathways:

$$J_{K^+OAc} = J_{HOAc} = J_{K/H} \quad (21)$$

The observed rate of KOAc transport can be expressed as follows

$$v = \frac{L'_{K/H} L'_{HOAc}}{L'_{K/H} + L'_{HOAc}} \quad (22)$$

As L_{K/H} is increased by addition of nigericin, v must approach a v_{max} defined by

$$v_{max} = L'_{HOAc} \quad (23)$$

K⁺/H⁺ antiport conductance must be proportional to the membrane concentration of nigericin, N, so that

$$L'_{K/H} = L'^0_{K/H} + QN \quad (24)$$

where Q is the turnover number (min⁻¹) of the K⁺-nigericin/H⁺-nigericin couple which obtains at the given gradient. L'^0_{K/H} describes endogenous conductance, if any, and may be eliminated using Equation 25:

$$L'^0_{K/H} = r v_0 \quad (25)$$

where

$$r = \frac{v_{max}}{v_{max} - v_0} \quad (26)$$

and v₀ is the rate in the absence of nigericin. Equation 22 may now be written as an Eadie-Hofstee plot

$$v = v_{max} \left\{ \frac{1 - r(v - v_0)}{QN} \right\} \quad (27)$$

Equation 27 provides a means for testing whether v_{max} or Q are affected by various treatment protocols. Equation 27 is independently valid, whatever the endogenous mechanisms for K⁺ transport. Thus, a plot of v versus (v - v₀)/N should be linear, and this is a test of the underlying assumptions and validity of approach.

Distinguishing K⁺ uniport from K⁺/H⁺ antiport during passive uptake of KOAc - Table I summarizes the experimental criteria for distinguishing endogenous K⁺ transport pathways. Given a high permeability to HOAc and the requirement for overall electroneutrality, spontaneous transport of KOAc can be mediated by electroneutral K⁺/H⁺ antiport (Cases A, B and C), by electrically coupled K⁺ and H⁺ uniport pathways (Case D) or by a combination of uniport and antiport (Case E). Under conditions where transport is mediated by K⁺/H⁺ antiport, a uniport pathway for K⁺ or H⁺ may exist in the membrane but cannot contribute to net transport in the absence of an electro-compensating pathway. The presence of such a non-contributing uniport pathway can, however, be revealed experimentally by providing the appropriate ionophore to complete the electrical couple. Thus, stimulation of endogenous rate of KOAc transport by valinomycin is diagnostic of a non-contributing H⁺ uniport pathway (Case C, Table I). Stimulation by CCCP indicates the presence of a silent K⁺ uniport pathway (Case B, Table I). In the absence of any endogenous uniport pathway, neither CCCP nor valinomycin can affect the rate of transport and therefore spontaneous transport can only be mediated by K⁺/H⁺ antiport and HOAc uniport (Case A, Table I).

Equations 16-20 are required if K⁺ uniport contributes to total transport (f ≠ 0). If KOAc transport is mediated by both pathways (Case D, Table I), then Equation 16 is used to determine the relative contribution of each. If KOAc transport is mediated solely by K⁺ uniport (L_{K/H} = 0, f = 1), a relationship must exist between the four observed rates, and it is given by

$$\frac{v_0}{v_N} = \frac{v_0}{v_V} + \frac{v_0}{v_C} - 1 \quad (28)$$

These considerations, summarized qualitatively in Table I, provide a simple and sensitive means for distinguishing K⁺ uniport from K⁺/H⁺ antiport during passive uptake of KOAc. The analysis is based on the assumptions that endogenous pathways exist which are competent to support net KOAc transport and that the membrane is permeable to HOAc. The diagnostics developed are mutually exclusive; therefore they should allow us to remove the ambiguity previously noted by Nakashima and Garlid (6) with respect to the underlying mechanism of K⁺ transport.

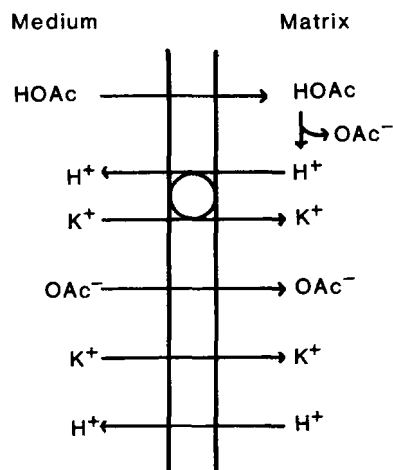


Fig. 1. Intrinsic ion transport pathways in the inner membrane of rat liver mitochondria. Shown are intrinsic pathways for net uptake of potassium acetate which are, in principle, physicochemically distinct and separable by experiment (See text). These include uniport pathways for H⁺ (or OH⁻) and OAc⁻ which, due to the high intrinsic permeability to HOAc, are not readily distinguished from each other but can be distinguished from electroneutral HOAc uniport. Carrier-mediated K⁺/H⁺ antiport is obligatorily electroneutral, while the K⁺ uniport pathway is independent of H⁺ uniport and coupled to it via electrical forces. Each of these pathways is passive, catalyzing transport from a bulk medium of high thermodynamic potential to one of low potential. The conductances of these pathways are evaluated in non-respiring mitochondria, in which the overall gradient driving potassium acetate transport is found experimentally to be inward.

Table I. Criteria for distinguishing between contributions from K⁺ uniport and K⁺/H⁺ antiport during transport of K⁺ acetate.

	INTRINSIC PATHWAYS		EXPERIMENTAL CRITERIA	
	contributing	non-contributing	valinomycin	CCCP
A. K ⁺ /H ⁺		none	v _V = v ₀	v _C = v ₀
B. K ⁺ /H ⁺		K ⁺	v _V = v ₀	v _N > v _C > v ₀
C. K ⁺ /H ⁺		H ⁺	v _N > v _V > v ₀	v _C = v ₀
D. K ⁺ and H ⁺		none	v _N > v _V > v ₀	v _N > v _C > v ₀
E. K ⁺ , H ⁺ and K ⁺ /H ⁺		none	v _N > v _V > v ₀	v _N > v _C > v ₀

All possible combinations are listed in which net, passive transport of KOAc is observed (See Fig. 1). It is given that electroneutral permeability to HOAc is high, so any K⁺/H⁺ antiport must contribute to net transport (Cases A-C). In order for K⁺ uniport to contribute to transport (Cases D and E), electroneutrality requires an electro-compensating H⁺ (or OAc⁻) uniport pathway. Thus, intrinsic uniport pathways may exist for K⁺ (Case B) and H⁺ (Case C), but they cannot contribute to net transport in the absence of a uniport pathway for their respective counterions.

Experimental criteria refer to observed rates in the absence of ionophores (v₀) and in the presence of saturating doses of nigericin (v_N), valinomycin (v_V) and CCCP (v_C). Cases D and E differ in the extent to which K⁺/H⁺ antiport contributes to transport and may be distinguished by the fact that the experimental rates in Case D (absence of intrinsic K⁺/H⁺ antiport) must obey Equation 28.