

Mechanisms for the Transport of α,ω -Dicarboxylates through the Mitochondrial Inner Membrane*

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α,ω -Dicarboxylates have antibacterial properties, have been used in the treatment of hyperpigmentary disorders, are active against various melanoma cell lines, and can also undergo β -oxidation. Little, however, is known about their transport. In this paper, we examine the mitochondrial transport of α,ω -dicarboxylates ranging from oxalate (DC2) to sebacate (DC10). DC2–DC10 are transported by the inner membrane anion channel (IMAC). DC6–DC10 are also transported by an electroneutral mechanism that appears to reflect transport of the acid through the lipid bilayer. At 37 °C and pH 7.0, DC10 is transported very rapidly at 3 $\mu\text{mol}/\text{min}\cdot\text{mg}$, and respiring mitochondria swell in the K^+ salts of these acids. This transport mechanism is probably the major pathway by which the longer dicarboxylates enter cells, bacteria, and mitochondria. We also demonstrate that DC5–DC10 can also be transported by an electroneutral mechanism mediated by tributyltin, a potent inhibitor of IMAC. The mechanism appears to involve electroneutral exchange of a TBT-dicarboxylate-H complex for TBT-OH. Finally, we present evidence that of all the dicarboxylates tested only DC2–DC4 can be transported by the classical dicarboxylate carrier.

α,ω -Dicarboxylates are physiologically and pharmacologically important compounds that arise naturally from the microsomal-initiated ω -oxidation of medium to long chain fatty acids (1, 2). Interest in these compounds arises for several reasons. First, they tend to accumulate and are excreted when normal fatty acid metabolism is disrupted, e.g. in diabetes, starvation, high fat diet, or when β -oxidation is defective (1, 3). Second, unlike medium chain monocarboxylates they are water-soluble, but like monocarboxylates they can be β -oxidized and, as a consequence, are potentially useful as parenteral nutrients (4). Third, at high concentrations they have been proved useful in the topical treatment of hyperpigmentary disorders of the skin (5–11) and also for the treatment of acne (11, 12–14).

β -Oxidation of dicarboxylates can take place in both mitochondria and peroxisomes (15, 16); however, many believe that in intact cells the peroxisomes are the primary site (17–21). The mechanism of transport of these acids into peroxisomes and mitochondria has not been established (15, 16, 19, 20); however, little attention has been paid to the possible role of

passive diffusion of the acid through the lipid bilayer.

In vitro, they have been shown to have antiproliferative and cytotoxic effects on cultured melanoma cell lines (5–9) and also antibacterial effects against microorganisms such as *Propionibacterium acnes* and *Staphylococcus epidermis* that are associated with acne (12–14). This latter property has led to the recent approval in the U. S. of an azelate-containing cream for the treatment of acne. For both of these actions, transport of the dicarboxylate into the cells appears to be important. Moreover, one of their major effects on tumor cells appears to be the induction of swelling and disruption of the mitochondria (5–7).

Our interest in the transport of α,ω -dicarboxylates arose from studies of the mitochondrial inner membrane anion channel (IMAC)¹ that mediates the transport of a wide variety of anions including several dicarboxylates (22). In a study of the selectivity of IMAC, we found that the transport of adipate (DC6) was much slower than that of malonate (DC3), while the transport of butylmalonate and 1,2,3-benzene-tricarboxylate were faster than malonate (22). Thus, the present study began with an investigation of the mechanism of this selectivity; however, it led to the identification and characterization of two previously undescribed pathways for the electroneutral transport of medium chain dicarboxylic acids through the lipid bilayer, one involving cotransport with protons that is probably important in their physiological and pharmacological roles and another mediated by tributyltin.

EXPERIMENTAL PROCEDURES

Assay of Anion Transport—Anion transport was assayed by following swelling that accompanies net salt transport, using the light scattering technique as described in detail elsewhere (23–25). Using this technique, we generate a light scattering variable, β , which normalizes reciprocal absorbance for mitochondrial protein concentration. The rate of salt transport is calculated from the rate of change of β . Although the contamination of mitochondrial preparations by peroxisomes can complicate metabolic measurements, peroxisomes make a negligible contribution to light scattering changes in mitochondrial preparations.

Pretreatment of Mitochondria for Assay of IMAC—The normal mitochondrial stock suspension (50 mg of protein/ml) was diluted 1:5 in K^+ MOPS (25 mM) and EDTA (5 mM) adjusted to pH 7.4 (at 25 °C) and maintained at 0 °C. A23187 (1 nmol/mg), nigericin (0.5 nmol/mg), and rotenone (0.5 $\mu\text{g}/\text{mg}$) were added, and at least 10 min was allowed to elapse after mixing before the mitochondria were transferred to the various assay media. To investigate inhibition of IMAC by DCCD, DCCD (50 nmol/mg) was also added to this medium and 45 min allowed to elapse before assay (26).

Pretreatment of Mitochondria for Assay of Non-IMAC-mediated Transport—The pretreatment was the same as described above, except A23187 and nigericin were omitted (control) and, where indicated, DCCD (50 nmol/mg) was added (+DCCD). Again 45 min was allowed to

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¹ The abbreviations used are: IMAC, inner membrane anion channel; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; DC2, oxalate; DC3, malonate; DC4, succinate; DC5, glutarate; DC6, adipate; DC7, pimelate; DC8, suberate; DC9, azelate; DC10, sebacate; TBT, tributyltin; TMPD, *N,N,N',N'*-tetramethylenephenediamine.

elapse before transport assays were begun to ensure complete inhibition of IMAC.

Assays of Succinate Transport (DC4)—Whenever succinate transport was assayed, the pretreatment medium was supplemented with antimycin A (0.2 nmol/mg) to block respiration.

Pretreatment of Mitochondria for Assay of TBT-mediated Transport—Mitochondria were pretreated as described above for assay of IMAC, except nigericin was omitted, the pH was raised to 7.8, and DCCD (50 nmol/mg) was added. Under these conditions, both IMAC and the endogenous K^+/H^+ antiporter are blocked by DCCD (26, 27).

These three pretreatments proved to yield stable preparations that could be used for several hours without a change in the rates of swelling.

Assay Media for Anion Transport—The media for assay of transport contained the K^+ salts of the appropriate dicarboxylate (36.7 mM) and EDTA (0.1 mM), EGTA (0.1 mM), and MOPS (5 mM). All media were made to the same tonicity (110 mOsm). For experiments in which the temperature was to be varied, the pH of the assay medium was first adjusted at 25 °C to a value, calculated on the basis of a value of -0.0095 for $\Delta pK^{\circ}C$ for the buffer, that would yield the desired pH at the desired temperature. In this way, separate assay media were prepared for each temperature to be studied. The value of $\Delta pK^{\circ}C$ was determined experimentally in the dicarboxylate assay medium described above. The temperature of each assay tube was measured prior to the addition of mitochondria to ensure a steady value had been achieved. For studies in which the effect of pH was to be investigated at a given temperature, separate media were prepared at approximately the desired pH, and the actual pH value of each assay tube was measured at the completion of each run while the assay tube remained in the water bath.

Drugs and Reagents—Most drugs were obtained from Sigma. The ionophores and rotenone were dissolved in ethanol. DCCD was dissolved in ethanol. Rat liver mitochondria were prepared as described previously (23).

RESULTS

Effect of Chain Length on Dicarboxylate Flux through IMAC—The mitochondrial inner membrane anion channel can be activated by depletion of endogenous Mg^{2+} using the ionophore A23187 (22). Thus, if valinomycin is added to provide an electrophoretic pathway for the influx of K^+ , when suspended in K^+ salts, Mg^{2+} -depleted mitochondria swell at a rate limited by the permeability to the anion. The data contained in Fig. 1A show typical traces obtained at pH 8.4 and 37 °C in potassium salts of dicarboxylates ranging from oxalate (DC2) to sebacate (DC10). The salt fluxes measured between β values of 0.32–0.38 are compared in Fig. 1B and show that the flux decreases as the chain length increases from DC2 to DC5. For DC5 to DC9, there is relatively little dependence of the rate on chain length. Fig. 1B also shows that the flux observed with butylmalonate under these conditions is close to that of malonate demonstrating that it is not the increase in size of the dicarboxylate *per se* that causes the decrease in rate with chain length. To verify that these fluxes were mediated by IMAC, a parallel experiment was carried out using mitochondria treated with DCCD to irreversibly inhibit IMAC (26). With these mitochondria the flux of DC2–DC9 was inhibited by greater than 99% (trace *j*, Fig. 1A) and the flux of DC10 by 93%.

Temperature Dependence of Dicarboxylate Transport through IMAC—Previously, we showed that the flux of malonate through IMAC is extremely temperature-dependent with the slope of the Arrhenius plot increasing from -20 kJ/mol at 40 °C to -190 kJ/mol at 5 °C (25). This change in slope was interpreted as a temperature-dependent transition from an active state to an inactive state of IMAC, *e.g.* a change in open probability as described by Equation 1:

$$\ln J = A + \ln T - \frac{\Delta H^{\ddagger}}{RT} + \frac{\Delta S^{\ddagger}}{R} - \ln \left(1 + \exp \frac{\Delta H_{\text{open}}}{R} \left(\frac{1}{T} - \frac{1}{T_{50}} \right) \right) \quad (\text{Eq. 1})$$

where ΔH^{\ddagger} and ΔS^{\ddagger} are the activation enthalpy and entropy, respectively, of transport; ΔH_{open} is the enthalpy of channel

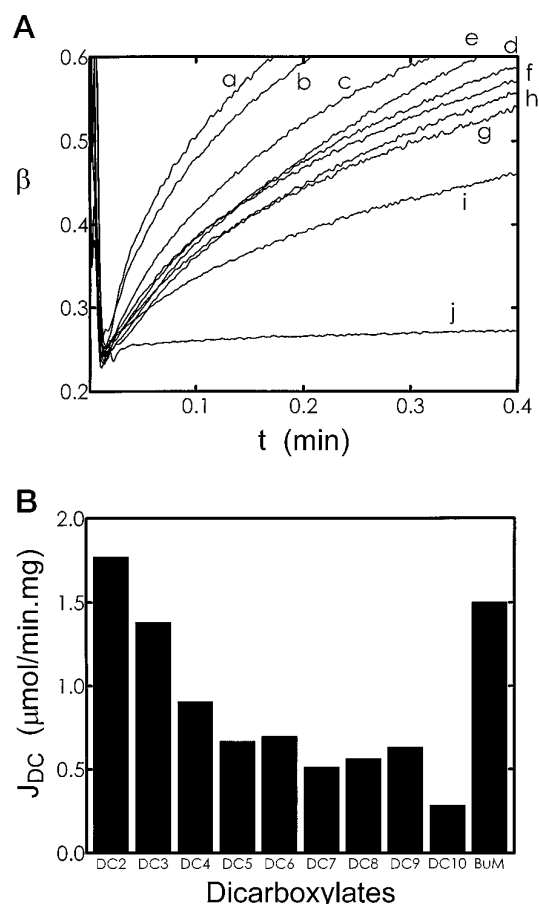


FIG. 1. Selectivity of IMAC for α,ω -dicarboxylates. A, light scattering kinetics of mitochondria (0.13 mg/ml) suspended in potassium dicarboxylate media at pH 8.4 and 37 °C are shown. The light scattering parameter β is a function of mitochondrial volume as described under "Experimental Procedures." The mitochondria were pretreated with A23187 (1 nmol/mg), nigericin (0.5 nmol/mg), and rotenone (0.5 μ g/mg). Valinomycin (0.5 nmol/mg) was added to the assay medium. Traces *a–j* represent the dicarboxylates DC2–DC10, respectively. Trace *j* shows effect of adding DCCD to the pretreatment in DC2 medium. B, the rates of dicarboxylate transport (J_{DC}) determined from the slopes measured between β values of 0.32–0.38 of the traces shown in A are compared. *BuM* is the rate of butylmalonate transport under similar conditions. The composition of the assay media and other experimental details are described under "Experimental Procedures."

opening; $T_{50} = \Delta H_{\text{open}}/\Delta S_{\text{open}}$; and T is the absolute temperature (25).

If this interpretation is correct each anion should exhibit the same characteristic temperature dependence. This is confirmed by the data in Fig. 2A; moreover, fitting Equation 1 to the data yields values of ΔH_{open} and T_{50} that are essentially independent of the chain length and rate of transport (Fig. 2B). Only with sebacate (DC10) is a significantly lower slope observed; however, this appears to be due to the existence of another transport pathway.

Dicarboxylates Can Also Be Transported Electroneutrally—When we examined the temperature dependence of the transport of the dicarboxylates at pH 7.4 (data not shown), we obtained data that suggested that the longer chain dicarboxylates were being transported by a pathway independent of IMAC. To investigate this possibility, we examined the transport of the dicarboxylates in the absence of valinomycin in non- Mg^{2+} -depleted mitochondria at pH 7.0, conditions designed to minimize the activity of IMAC. Typical traces obtained at 37 °C are shown in Fig. 3A, and as shown in Fig. 3B, the rate of transport increases exponentially from 9 nmol/

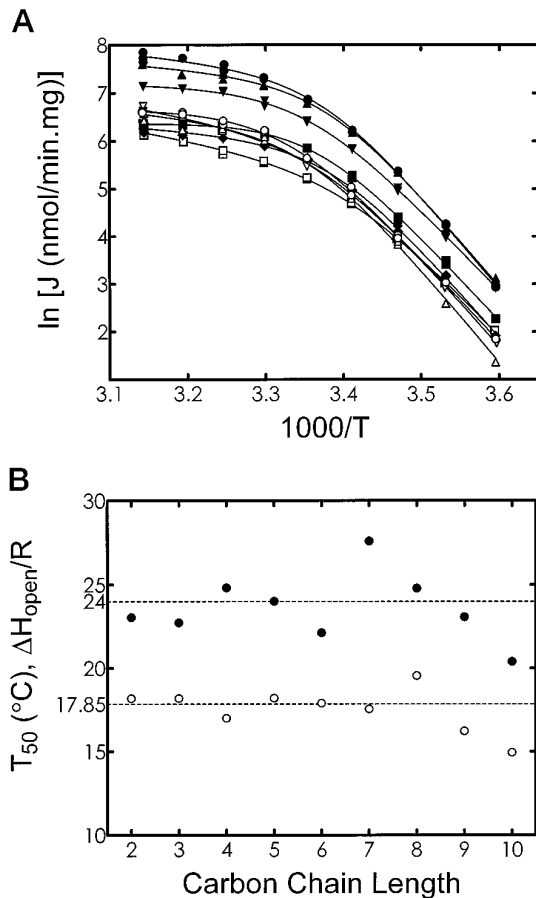


FIG. 2. Temperature dependence of dicarboxylate transport via IMAC. Fluxes were measured for each of the nine dicarboxylates as described in Fig. 1 at a series of temperatures ranging from 5 to 45 $^{\circ}\text{C}$. A, the Arrhenius plot for each anion is shown: \bullet , DC2; \blacktriangle , DC3; \blacktriangledown , DC4; \blacksquare , DC5; \blacklozenge , DC6; \circ , DC7; \triangle , DC8; ∇ , DC9; \square , DC10. The curves were fitted to the data using Equation 1. B, values of $\Delta H_{\text{open}}/R$ (\circ) and T_{50} (\bullet) derived from the fit of Equation 1 to the data in A are plotted versus the carbon chain length of the dicarboxylate. The dotted lines show the mean values determined for DC2–DC9. See “Experimental Procedures” for further details.

min·mg for adipate to 2900 nmol/min·mg for sebacate. Unlike the fluxes shown in Fig. 1, these fluxes are not blocked by pretreatment of the mitochondria with DCCD (Fig. 3B); moreover, they are not affected by depletion of matrix Mg^{2+} nor by the addition of valinomycin (not shown); thus these fluxes are independent of IMAC. Since only nigericin, a K^+/H^+ antiporter is necessary to permit these rapid salt fluxes, it is concluded that these dicarboxylates are transported via an electroneutral mechanism involving the cotransport of protons.

pH Dependence of the Electroneutral Transport of Dicarboxylates—To examine the relationship between the flux and pH, we used azelate rather than sebacate and carried out the experiment at 10 $^{\circ}\text{C}$ in order to slow down the fluxes and facilitate the measurement of initial rates. The results of this experiment are contained in Fig. 4 and show that the flux increases from 4 nmol/min·mg at pH 7.6 to 300 nmol/min·mg at pH 6.65. The curve fitted to the data is described by Equation 2:

$$J = A \cdot 10^{-2\text{pH}} \quad (\text{Eq. 2})$$

which assumes two protons are involved in the transport process ($A = 7.48 \times 10^6 \text{ mol/min}\cdot\text{mg}\cdot\text{M}^2$). The dotted line shows the best fit obtained if it is assumed that only one proton is involved.

Temperature Dependence of Electroneutral Dicarboxylate

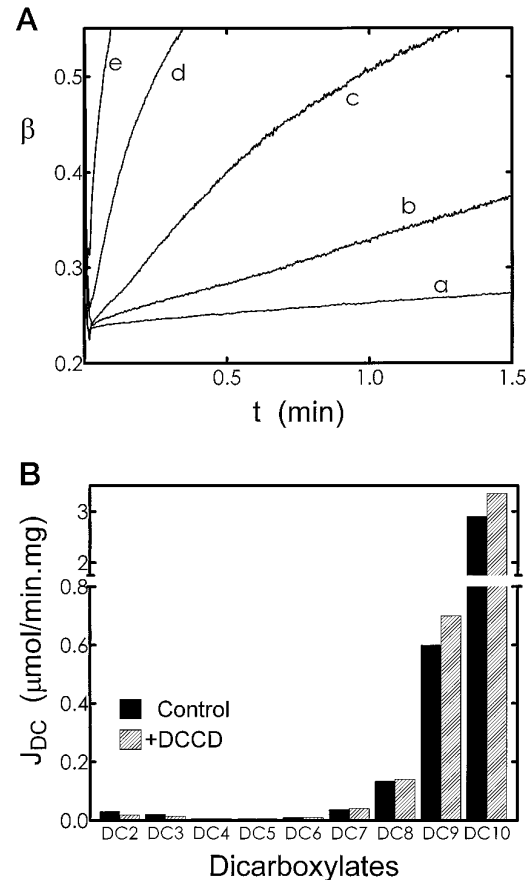


FIG. 3. Medium chain dicarboxylates can be transported electroneutrally. A, typical light scattering traces are presented for DCCD-treated mitochondria (0.12 mg/ml) suspended in potassium dicarboxylate media at pH 7.0 and 37 $^{\circ}\text{C}$. Nigericin (6 nmol/mg) and rotenone (2 $\mu\text{g}/\text{mg}$) were included in the medium, and the mitochondria were added at zero time. Trace a, DC6; b, DC7; c, DC8; d, DC9; e, DC10. B, rates of dicarboxylate flux determined in DCCD-treated and control mitochondria from traces similar to those shown in A are compared for the nine dicarboxylates. For further details see “Experimental Procedures.”

Transport—To further characterize this transport process and to allow estimation of the magnitude of fluxes at more physiological temperatures, we examined the temperature dependence of the electroneutral transport at pH 7.0 for dicarboxylates ranging from adipate to sebacate. The data contained in Fig. 5 are those obtained with DCCD-treated mitochondria; however, similar results were obtained in the absence of DCCD. The results show that unlike transport through IMAC, the electroneutral flux is characterized by a linear Arrhenius plot from 5 to 45 $^{\circ}\text{C}$. For each anion, the activation energy is close to 51.4 kJ/mol ($Q_{10} = 2.0$), and the increase in flux with chain length is most easily explained by an increase in the activation entropy (ΔS^{\ddagger}) of transport. From these results it is also evident that the log of the flux increases linearly with the chain length that suggests that the flux may be a simple function of the oil:water partition coefficient.

Electroneutral Dicarboxylate Transport Is a Function of the Partition Coefficient—To examine how closely the chain length dependence of the dicarboxylate flux may be attributed to the change in oil:water partition coefficient, in Fig. 6 we have plotted the log of the flux versus the number of carbons in the chain together with literature values of partition coefficients for series of linear organic alkanes, alcohols, and monocarboxylic acids in both octanol and hexadecane (28). For each series, a linear relationship is obtained with slopes ranging from 0.54

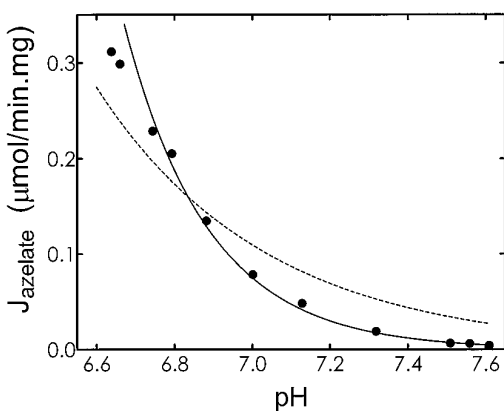


FIG. 4. **pH dependence of electroneutral dicarboxylate transport.** The rate of azelate flux (J_{azelate}) for DCCD-treated mitochondria suspended in potassium azelate assay medium at 10 °C is plotted versus the pH of the assay medium. The *solid line* was fitted to the data assuming that $J = A \cdot [\text{H}^+]^2$ ($A = 7.48 \times 10^6$ mol/min·mg·M²), whereas the *dotted line* was fitted assuming that $J = A \cdot [\text{H}^+]$. The measurements were made as described in Fig. 3. See "Experimental Procedures" for further details.

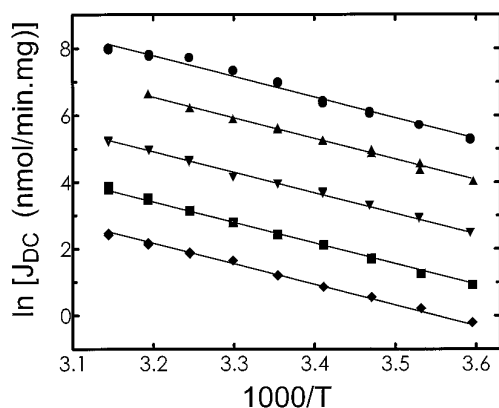


FIG. 5. **Temperature dependence of electroneutral dicarboxylate transport.** Arrhenius plots are shown for fluxes determined as described in Fig. 3 utilizing DCCD-treated mitochondria in assay media maintained at pH 7.0 as described under "Experimental Procedures." The temperature was varied from 5 to 45 °C. ●, DC10; ▲, DC9; ▼, DC8; ■, DC7; ◆, DC6. The lines were fitted assuming a constant slope = 6.178K (=51.36 kJ/mol) equal to the mean value for the five dicarboxylates. See "Experimental Procedures" for further details.

to 0.67. The dicarboxylate fluxes yield a slope of 0.63, equivalent to an increase in activation free entropy of 12 J/mol·K per $-\text{CH}_2-$ with a 4.25-fold increase in rate per $-\text{CH}_2-$. Thus, the increase in electroneutral anion flux with chain length is directly proportional to the concentration of the acid in the bilayer. From these findings, we conclude that the electroneutral flux occurs through the lipid bilayer.

Uptake of Dicarboxylates Can Be Driven by Respiration—Energized mitochondria generate a high membrane potential that drives the electrophoretic influx of K^+ . In the steady state this flux is compensated by electroneutral efflux of K^+ by the K^+/H^+ antiporter; however, when anions of permeant acids, such as acetate are added, the gradient for K^+/H^+ declines and respiration drives net influx of salt with consequent osmotic swelling (29). Thus, if the dicarboxylic acids are permeant acids they should also be able to support respiration-dependent osmotic swelling, and this could explain the mitochondrial damage observed in tumor cells treated with azelate (5–7, 9). The data in Fig. 7A confirm this prediction. As expected (29), mitochondria respiring on ascorbate/TMPD swell in media containing acetate (*trace a*) but not in pure KCl (*trace f*). They also

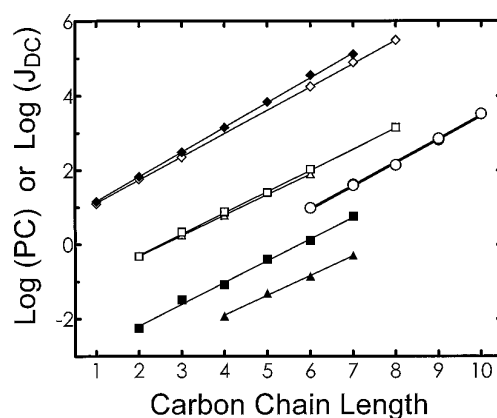


FIG. 6. **The relationship between electroneutral dicarboxylate flux, chain length, and partition coefficients.** The log of the dicarboxylate flux determined at 37 °C as described in Fig. 3 ($\log(J_{\text{DC}})$, 0)) is plotted versus the carbon chain length for DC6–DC10. The slope = 1.45 indicates that the activation free energy decreases 3.7 kJ/mol per $-\text{CH}_2-$. For comparison, the relationship between the log of the hexadecane:water (*closed symbol*) and octanol:water (*open symbol*) partition coefficients (PC) for *n*-alkanes ◆, ◇; *n*-alkyl alcohols ■, □; and *n*-alkyl monocarboxylic acids ▲, △ reported in Ref. 28 are shown.

swell if the acetate is replaced by DC10, DC9, or DC8 (*traces b*, *c*, and *d*) but not with DC6 (*trace e*). Although these rates are to some extent limited by the K^+ permeability, the rates of swelling are found to follow the permeability sequence observed in Fig. 3. The data shown in Fig. 7B show that these fluxes are coupled to respiration. No swelling is observed if the substrates are omitted (*trace b*), and swelling is almost completely inhibited by addition of the K^+/H^+ exchanger nigericin (*trace c*) or the protonophore carbonyl cyanide *p*-chlorophenylhydrazone (*trace e*). The residual flux in the presence of these ionophores is not dependent on respiration, since in the presence of the ionophores omission of substrates (*traces d* and *f*) has a negligible effect.

Tributyltin Mediates Transport of Dicarboxylates through Lipid Bilayers—In the studies discussed above, we employed DCCD to help distinguish the electrophoretic transport of dicarboxylates through IMAC from their electroneutral transport through the bilayer. Before choosing DCCD, however, we tested tributyltin that is probably the most potent and selective inhibitor of IMAC identified to date (30). Even at alkaline pH, however, TBT could not completely block the flux of azelate or sebacate in our IMAC assay; moreover, increasing the dose only increased the rate. Since it has been established that TBT and other trialkyltins can mediate electroneutral Cl^-/OH^- exchange across membranes (31), we investigated whether a similar phenomenon was occurring with the dicarboxylates.

In the presence of nigericin, TBT induces net flux of potassium azelate in DCCD-treated mitochondria. Unlike transport through IMAC, this flux is not stimulated by valinomycin, and unlike the electroneutral transport discussed above, significant rates are observed at alkaline pH (not shown). Thus, since we have no way of inhibiting the TBT-independent electroneutral pathway, to characterize the TBT-dependent transport, we made measurements at pH 8.4 using DCCD-treated mitochondria. The pretreatment was modified slightly from that used for the assay of IMAC, by raising the pH to 7.8 in order to block the mitochondrial K^+/H^+ antiporter (27). With the K^+/H^+ antiporter blocked, we find that the TBT-induced flux is almost completely dependent on the presence of nigericin and, therefore, that TBT does not induce nonselective leakage.

The data contained in Fig. 8 demonstrate that in the range from 1 to 12 nmol/mg the net flux is linearly dependent on the concentration of TBT. The slope yields a turnover number of

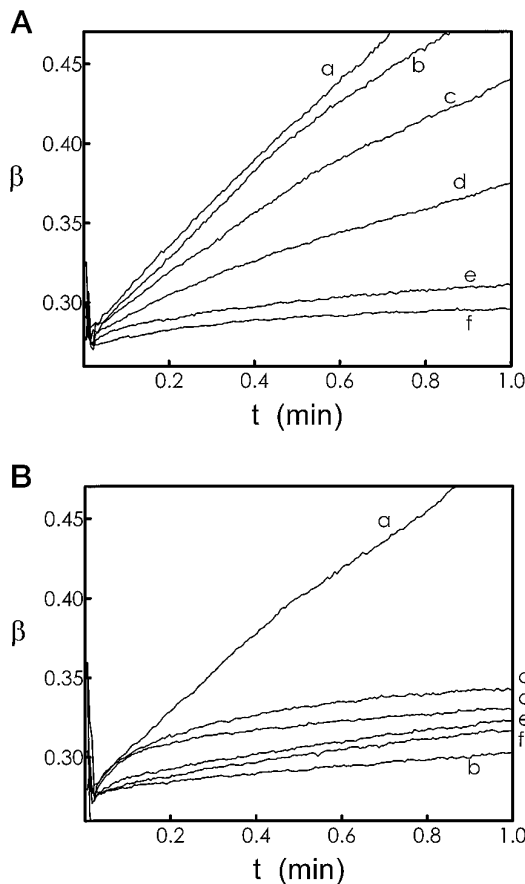


FIG. 7. Uptake of dicarboxylates can be driven by respiration. Light scattering kinetics of mitochondria (0.14 mg/ml) suspended in assay media containing mixtures of potassium salts at pH 7.0 and 37 °C are shown. *A*, the media contained the respiratory substrates ascorbate (1.5 mM), TMPD (0.15 mM), cytochrome *c* (10 μ M) plus $MgCl_2$ (0.1 mM), EGTA (0.1 mM), and MOPS (5 mM), plus the following K^+ salts: *trace a*, acetate (30 mM), Cl^- (45 mM); *trace b*, sebacate (20 mM), Cl^- (45 mM); *trace c*, azelate (20 mM), Cl^- (45 mM); *trace d*, suberate (20 mM), Cl^- (45 mM); *trace e*, adipate (20 mM), Cl^- (45 mM); *trace f*, Cl^- (75 mM). *B*, all traces were obtained in the azelate/ Cl^- medium. *Trace a*, control (plus ascorbate/TMPD); *trace b*, no ascorbate/TMPD; *trace c*, ascorbate/TMPD, plus nigericin (2.5 nmol/mg); *trace d*, no ascorbate/TMPD, plus nigericin; *trace e*, ascorbate/TMPD, plus carbonyl cyanide *p*-chlorophenylhydrazone (8 μ M); *trace f*, no ascorbate/TMPD, plus carbonyl cyanide *p*-chlorophenylhydrazone. In all experiments, the mitochondria were pretreated as described in Ref. 29 with oligomycin (1.5 nmol/mg) and rotenone (2 μ g/mg).

about 71 min^{-1} . This compares with a value of 260 min^{-1} for Cl^- transport at 25 °C (30). The linearity of this relationship strongly suggests that TBT is mediating the transport and is not simply activating another transport pathway. The positive intercept on the abscissa (0.8 nmol/mg) is the same as that observed for TBT-induced Cl^-/OH^- antiport (30) and is close to the amount needed to block IMAC and the F_1F_0 -ATPase and, therefore, probably reflects the amount of TBT bound tightly to mitochondrial proteins.

The data contained in Fig. 9, show the relationship between the TBT-induced flux and the chain length of the dicarboxylates. Consistent with a mechanism involving complex formation between TBT and the dicarboxylate in the bilayer, the flux increases with the chain length. The slope of the semi-log plot is, however, less than that observed in the absence of TBT yielding a 2.2-fold increase in rate per $-CH_2-$ that is equivalent to an increase in ΔS^\ddagger of 6.6 J/mol·K per $-CH_2-$. This suggests that the partition coefficient of the dicarboxylates is less critical to the transport mechanism.

Transport of Dicarboxylates through the Dicarboxylate Car-

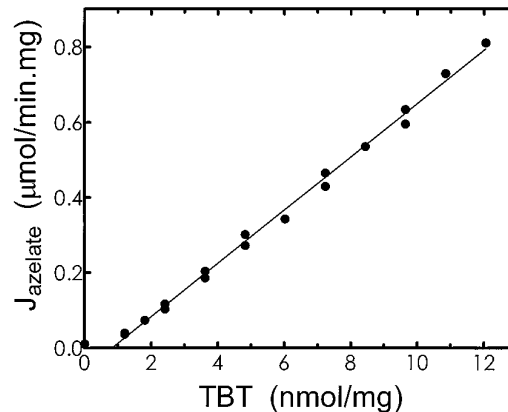


FIG. 8. TBT dependence of electroneutral azelate flux. The rate of azelate flux is plotted *versus* the dose of TBT. The slope of the curve fitted to the data = 71 min^{-1} . For further details see "Experimental Procedures."

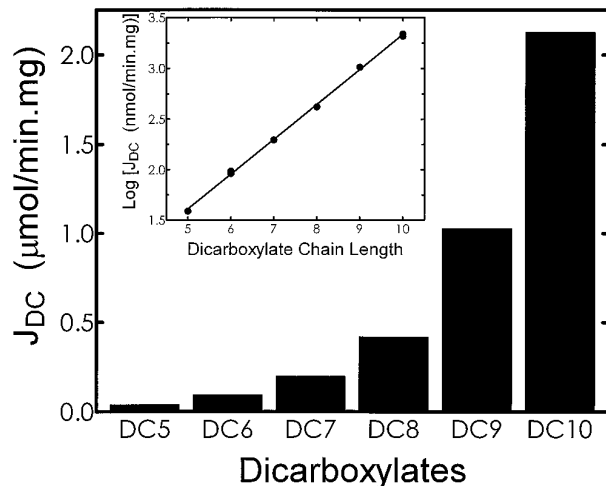


FIG. 9. Relationship between TBT-induced dicarboxylate flux and chain length. Rates of dicarboxylate flux induced by TBT (12 nmol/mg) determined as described in Fig. 7 are compared. The *inset* shows the relationship between the log of the flux and the chain length. The slope 0.34 yields a value of 2.0 kJ/mol $\times -CH_2-$ for the decrease in activation energy with chain length. See "Experimental Procedures" for further details.

rier—To complete our study of dicarboxylate transport mechanisms in mitochondria, we carried out assays to determine which dicarboxylates can be transported by the classical dicarboxylate carrier. This carrier mediates electroneutral exchange of dicarboxylates as well as electroneutral exchange of dicarboxylates for P_i . Thus, addition of catalytic concentrations of P_i permits net influx of K^+ dicarboxylates in the presence of nigericin. In Fig. 10, the effect of P_i on electroneutral dicarboxylate flux is shown for DC2 to DC10 in DCCD-treated mitochondria. Only with oxalate, malonate, and succinate is stimulation seen. Thus, α,ω -dicarboxylates with five carbons or more do not appear to be substrates for the dicarboxylate carrier. These data also demonstrate that the rates of DC8–DC10 electroneutral transport possible under these conditions can be as fast if not faster than carrier-mediated transport under physiological conditions.

DISCUSSION

In this paper, we have demonstrated that at least four pathways exist for the transport of dicarboxylates in mitochondria. These are electrophoretic transport via IMAC, electroneutral transport of the acid, TBT-mediated transport of the acid, and transport via the dicarboxylate carrier.

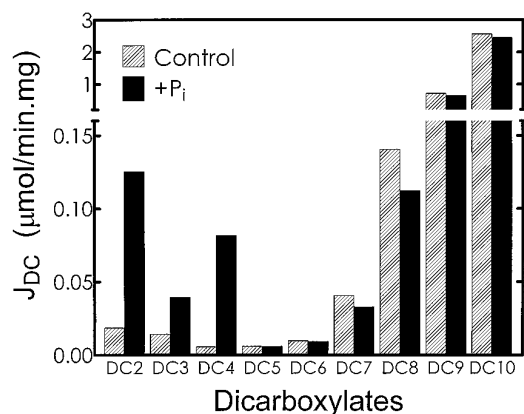


FIG. 10. **Selectivity of the dicarboxylate carrier for dicarboxylates.** Rates of dicarboxylate fluxes in DCCD-treated mitochondria (0.12 mg/ml) suspended in the dicarboxylate media containing nigericin (6 nmol/mg) at pH 7.0 and 37 °C in the presence and absence of phosphate are shown. Since net dicarboxylate flux through the dicarboxylate carrier requires the presence of phosphate, stimulation by P_i is assumed to reflect activity of the dicarboxylate carrier. When present, nigericin (6 nmol/mg) and P_i (2 mM) were added at zero time. See "Experimental Procedures" for further details.

Of the four pathways, IMAC displays the least selectivity, with only a 3-fold difference in flux between oxalate and azelate at pH 8.4. Fluxes of all the dicarboxylates are irreversibly inhibited by DCCD and reversibly inhibited by Mg²⁺ and protons. Moreover, they all have the same characteristic temperature dependence (25). This suggests that the decrease in flux observed as the chain length increases from oxalate to glutarate reflects a difference in permeability/conductance, and unlike the effect of pH or *N*-ethylmaleimide (25), it is not related to any change in the temperature dependence. The finding that the slopes of the steep segments of the Arrhenius plots and the values for *T*₅₀ are independent of the anion species supports our previous conclusion that IMAC undergoes a temperature-dependent activation (change in open probability).

One reason we began the present study was the observation that at pH 7.4 the transport rate of adipate through IMAC was much lower than that of malonate, butylmalonate, and 1,2,3-benzene-tricarboxylate (22). Thus, it appeared that the distance between the charges in adipate may be responsible for the slow transport. The additional recent observation that 4,4-diisothiocyanostilbene-2,2'-disulfonic acid, which contains 6 carbons between the two sulfonate groups, inhibits IMAC (32) led us to hypothesize that longer chain dicarboxylates may be better inhibitors or more slowly transported. Our findings presented here indicate that this is not the case. We find no significant difference in fluxes as the chain length increases from DC5 to DC9. Instead, we find that the rate increases as the chain length decreases from DC5 to DC2. Thus, we believe that it is the proximity of the charges, rather than the size of the anion or the chain length, that determines its rate of flux. The transport mechanism probably involves interaction of the two charges at a specific site in the channel. As the chain length increases, the benefit of the proximity of the two charges is gradually lost. This finding suggests that this selectivity filter, although weak, is similar to that proposed for the dicarboxylate carrier (33). Other examples of similarities between IMAC and the dicarboxylate carrier have been reported (25, 34). In view of the report by Jezek and Garlid (35), that the transport rates of alkyl-sulfonates through IMAC are independent of chain length, it is possible that the interaction of the longer dicarboxylates with IMAC is similar to that of monocarboxylates.

The second pathway for dicarboxylate transport we have studied has not been previously described. Its properties differ from those of IMAC in almost every way. It mediates electroneutral transport, and its selectivity is opposite and much greater than that of IMAC. It is activated, not inhibited by protons. It has a linear Arrhenius plot and it is not inhibited by DCCD or Mg²⁺. Thus, the two pathways are readily distinguishable.

The following properties lead us to conclude that this electroneutral flux represents passive diffusion of the acid through the lipid bilayer. First, the flux increases with chain length in parallel with the published *n*-hexadecane:water and *n*-octanol:water partition coefficients of related compounds. Second, the relationship between the flux and [H⁺]² is almost linear. Third, like other compounds believed to permeate lipid bilayers, including NH₄OAc, erythritol, malonamide, SCN⁻ (25), this transport process exhibits a linear Arrhenius plot with a *Q*₁₀ close to 2.0. There is no significant change in the slope of the Arrhenius plot from DC6 to DC10; consequently, it is concluded that the activation enthalpy for transport (ΔH^\ddagger) does not vary among these dicarboxylates and that the increase in flux with chain length results from an increase in the activation entropy (ΔS^\ddagger). Furthermore, since this increase, equal to 12 J/mol·K per —CH₂— (3.7 kJ/mol at 37 °C), parallels the increase in the partition coefficients of related compounds, we conclude that it reflects an increase in the partitioning of the dicarboxylates into the lipid bilayer that is an entropy-driven process. Further evidence for passive diffusion of the acid comes from the finding that respiring mitochondria can drive the uptake of potassium suberate, azelate, and sebacate. In this process, K⁺ uptake driven by the membrane potential is mediated by electrophoretic pathways including an ATP-sensitive K⁺ channel (29). This mechanism is most probably responsible for the mitochondrial swelling observed in a variety of cultured tumor cells in the presence of high concentrations of azelate (5–7, 9). The existence of both an electroneutral proton symport mechanism and an electrophoretic mechanism (IMAC) for the transport of these dicarboxylates also allows for the futile cycling of these anions. The resulting uncoupling of respiring mitochondria by this mechanism could contribute to their antimitochondrial properties (5–7).

Passive influx of dicarboxylic acids into cells by electroneutral diffusion provides an explanation for why the longer dicarboxylates can be used as parenteral nutrients (4). In order to be β -oxidized by mitochondria or peroxisomes, they must first enter the cells. Studies have shown that the extent of oxidation increases as the chain length increases (4). Moreover, the percent of an IV infusion excreted in the urine decreases from 50% for azelate (DC9) to 12% for sebacate (DC10) to 1.6% for dodecanedioate (DC12) (4). These findings are consistent with our observation that the membrane permeability increases exponentially with chain length. Thus, as the chain length increases, the dicarboxylates will be taken up more rapidly by cells and organelles, and the extent of tubular reabsorption in the kidney will increase.

The extent to which dicarboxylates are oxidized by peroxisomes *versus* mitochondria in the intact cell has been the subject of a number of studies and remains controversial (15–21, 36). Most groups, however, have come to the conclusion that the peroxisomes are the major site of β -oxidation for dicarboxylates with 8 or more carbons. Little attention, however, has been paid to the transport mechanisms involved. It has been concluded that exogenous shorter chain dicarboxylates DC6–DC8 are not oxidized by mitochondria due to an inability to enter the mitochondria (15) and that the only mechanism by which the longer dicarboxylates DC10–DC12 enter the mito-

chondria is via the carnitine shuttle (15). Although this may in fact be the preferred pathway for dicarboxylates entering the β -oxidation pathway, *e.g.* the intramitochondrial CoA derivative may only be synthesized rapidly from the carnitine derivative, the data presented here clearly show that dicarboxylates DC9 and longer can rapidly enter mitochondria.

Another area of intense interest in dicarboxylates has focused on azelate (DC9) and its role in the treatment of acne, hyperpigmentary disorders, and its effect on cultured cancer cells (see Ref. 11 for a review). The antipigmentary effects are reported to result from inhibition of tyrosinase the key enzyme in melanin synthesis (8), but azelate also inhibits thioredoxin reductase, an enzyme involved in the synthesis of deoxyribonucleotides (11), and dicarboxylates are also reported to inhibit components of the mitochondrial respiratory chain (5, 7). Moreover, the mitochondria in cultured cells treated with high concentrations of azelate become swollen, and those in melanoma cell lines appear to be more sensitive than those in normal cells (6, 7, 9). The bacteriostatic effects on cutaneous microorganisms are reported to be enhanced by acidic pH (12, 13), and it has been suggested that they may be related to a decrease in the internal pH due to entry of the acid (14). Although the mechanism of dicarboxylate transport across the plasma membrane has not been established, the published observations suggest that passive diffusion of the acid through the bilayer is the major mechanism of transport through both the plasma membrane and the inner mitochondrial membrane as well as bacterial membranes. We believe that all the effects of azelate discussed above are dependent on the electroneutral transport mechanism described in this paper. Entry of dicarboxylates into cells by this mechanism will certainly be favored by low extracellular pH, and at high dicarboxylate concentrations, this will undoubtedly decrease the cytosolic and intramitochondrial pH and activate pH regulatory mechanisms such as the plasma membrane Na^+/H^+ antiporter. A decrease in mitochondrial matrix pH could lead to net influx of K^+ via K^+ channels as illustrated by our data or influx of Na^+ via the unregulated mitochondrial Na^+/H^+ antiporter, if cytosolic Na^+ levels were to rise. The apparent greater sensitivity of melanoma mitochondria to azelate suggests that their regulatory mechanisms may differ from those in normal cells.

The third dicarboxylate transport mechanism we have examined requires the presence of tributyltin. Although not tested, other trialkyltins probably behave in a similar manner. It has been established that these compounds are able to mediate exchange of Cl^- , Br^- , I^- , OH^- across lipid membranes (31); however, to our knowledge the ability of carboxylates to be transported by this mechanism has not been previously demonstrated. Since we observe a simple linear relationship between this flux and TBT concentration, the simplest mechanism to explain the observations is one in which TBT complexes one end of the dicarboxylate and a proton binds to the other thus forming a lipophilic neutral complex that can cross the bilayer.

Finally, we have presented evidence that only oxalate, malonate, and succinate can be transported via the dicarboxylate carrier. These findings confirm the reports by Strzelecki *et al.* (37, 38) that oxalate can be transported by the dicarboxylate carrier in liver and kidney mitochondria. We found no evidence for phosphate-dependent transport of the longer acids.

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