Citrate and Succinate Uptake by Potato Mitochondria

Received for publication June 26, 1978 and in revised form November 9, 1978

DENNIS W. JUNG and GEORGE G. LATIES
Department of Biology and Molecular Biology Institute, University of California, Los Angeles, California 90024

ABSTRACT

The uptake of [14C]citrate and [14C]succinate was studied in potato mitochondria (Solanum tuberosum var. Russet Burbank) using cellulose pore filtration and was found to occur by the same mechanisms as described for mammalian mitochondria. Potato mitochondria, in the absence of respiration, have a very low capacity for uptake by exchange with endogenous anions, taking up only 2.4 nanomoles citrate and 2.0 nanomoles succinate per milligram protein. Maximum citrate uptake of over 17 nanomoles per milligram protein occurs in the presence of inorganic phosphate, a dicarboxylic acid, and an external energy source (NADH), conditions where net anion accumulation proceeds, mediated by the interlinking of the inorganic phosphate, dicarboxylate, and tricarboxylate carriers. Maximum succinate uptake in the absence of respiratory inhibitors requires only added inorganic phosphate.

Compounds which inhibit respiration (antimycin), the exchange potential (mersalyl and benzylmalonate), or the establishment of the membrane proton motive force (uncouplers) reduce substrate accumulation. A potent inhibitor of the citrate carrier in animal mitochondria, 1,2,3-benzenetricarboxylic acid, does not inhibit citrate uptake in potato mitochondria. Citrate uptake is reduced by concurrent ADP phosphorylation and this reduction is sensitive to oligomycin. The initiation of state 3 after a 3-minute substrate state results in a reduction of the steady-state of citrate uptake by approximately 50%. Accumulation of succinate initially is inhibited by increasing sucrose concentration in the reaction medium from 50 to 400 millimolar.

Limited substrate uptake is one of the factors responsible for the often observed depressed initial state 3 respiration rates in many mitochondrial preparations. Since nonlimiting levels of substrate in the matrix cannot be attained by energy-independent exchange, a dependence on respiration for adequate uptake results. Substrate limitation therefore occurs in the matrix for the period of time needed for energy-dependent accumulation of nonlimiting levels.

Substrate anion transport occurs in mitochondria via a series of exchange carriers spanning the inner membrane (10, 37, 39). Mitochondria can take up citrate via the tricarboxylate carrier by exchange-diffusion with intramitochondrial di- or tricarboxylates in an energy-independent process. In the presence of an energy source net anion accumulation can occur since the proton gradient generated from respiration can be used for Pi/hydroxyl exchange, followed by dicarboxylate/Pi exchange, and citrate/dicarboxylate exchange with net citrate accumulation (24, 32). Transport of anions on the Pi, dicarboxylate and tricarboxylate carriers is electroneutral although that on the Pi and tricarboxylate carriers is associated with simultaneous proton movements, and exchange on these carriers is therefore dependent on the ΔpH across the inner mitochondrial membrane (24). Transport on the dicarboxylate carrier also becomes sensitive to ΔpH when anion flux involves it and another carrier which is proton compensated, such as the Pi or tricarboxylate carriers. Extensive characterization of these transport systems in mammalian mitochondria has been effected, and reports of the isolation and purification of the carriers have recently appeared (1, 30).

Although few in number, and limited mostly to related oxidation and swelling measurements, studies on the carriers in plant mitochondria have been in general agreement with the animal literature with the exception of glutamate transport (6-9, 33, 38, 39). DeSantis et al. (9) have recently used the exchange methods developed in studies on rat liver mitochondria to study transport in bean mitochondria. On the basis of studies of swelling in ammonium salts, potato mitochondria have been shown to possess Pi/hydroxyl, dicarboxylate and tricarboxylate carriers (33). The operation of these translocators is diagramed in Figure 7.

This study was initiated to investigate the relation between substrate uptake and oxidation in potato mitochondria, particularly in regard to conditioning, the phenomenon observed with many mitochondrial preparations of consecutive increasing state 3 respiration rates during several state 3/state 4 cycles (20, 35, 36). Our findings on the direct estimation of uptake using cellulose pore filtration indicate citrate and succinate uptake involves the same processes found for mammalian mitochondria. Potato mitochondria appear to be depleted of exchangeable anions, which results in a low capacity for uptake in the absence of respiration. Respiration allows net anion accumulation to occur and large amounts of citrate or succinate to be taken up. Respiration profoundly affects substrate uptake and this dependence of uptake on respiration is probably the major factor involved in conditioning.

MATERIALS AND METHODS

Mitochondria were isolated as previously described (18) from untreated potato tubers (Solanum tuberosum var. Russet Burbank) generously provided by Professor Herman Timm, Dept. of Vegetable Crops, University of California, Davis. Isolated mitochondria displayed good respiratory control and ADP/O ratios (18, 20).

Millipore filtration was used to measure the uptake of succinate or citrate. Mitochondria were incubated in a standard reaction mix of 200 mM sucrose, 1 mg/ml BSA (fraction V), 5 mM MgCl2, and 20 mM Tes (neutralized to pH 7.5 with KOH) at 25 C with other additions as specified. [1,4-14C]Succinate or [1,5-13C]Citrate (ICN, Irvine, Calif.) was added 1 min after the mitochondria. At various times, 0.2-ml aliquots of the reaction medium were pipetted into 3 ml of cold wash mix (200 mM sucrose, 20 mM Tes, pH

---

1 This work was supported by Energy Research and Development Administration Grant EY-76-S-03-0034.

2 Present address: Department of Physiological Chemistry, Ohio State University, Columbus, Ohio 43210.

7 The steady-state terminology follows that of Chance and Williams as modified by Bonner for plant mitochondria (2).

---

Downloaded from on December 15, 2017 - Published by www.plantphysiol.org
Copyright © 1979 American Society of Plant Biologists. All rights reserved.
7.5, 25 μM mersalyl, 10 mM benzylmalonate) placed over an 0.8-
μm filter in the chimney of a Millipore filter apparatus (Millipore
Corp., Bedford, Mass.). The absorption period was terminated by
starting the vacuum. Mersalyl and benzylmalonate serve to mini-
mize uptake during the wash period (see below). Filtration was
complete in 3 s. The underlayer of cold wash mix resulted in an
even spreading of the mitochondria over the filter and less varia-
bility. The wash mix was filtered directly to the filter.
Filters were washed once with 3 ml wash mix, removed, dried at
28 C, and placed in vials with 10 ml scintillation fluid (4 g PPO
and 95 mg POPP per liter toluene). The wash removed approxi-
amately 95% of the substrate nonspecifically bound to the filter,
and although it also removed some substrate from the mitochon-
dria, leading to underestimates of the amount taken up, it provided
more reproducible results due to lower backgrounds. Amounts
absorbed represent the total amount taken up in an experiment
minus that retained by the filter in the absence of mitochondria.
Benzylmalonic acid and BTCA4 were obtained from Aldrich
Chem. Co. (Milwaukee, Wis.). Protein was determined by the
method of Lowry et al. (22) and Pi was determined by the
isobutanol-benzene method of Lindberg and Ernster (21).

RESULTS

Dependence of Uptake on Citrate and Succinate Concentration.
The dependence on concentration of citrate and succinate uptake
in the presence and absence of 5 mM Pi is shown in Figure 1.
Arsenite was added in citrate experiments to block α-ketoglutarate
oxidation and thus prevent the loss of label as CO2. Arsenite
decreased the uptake of citrate. The citrate uptake isotherms
appear sigmoid whereas those for succinate tend more toward the
hyperbolic. In each case Pi consistently inhibits uptake to a small
extent at low substrate levels but stimulates uptake at higher
levels. The level of inhibition by Pi varied between preparations
and is more evident in the experiment using 0.5 mM citrate in
Table I. The stimulation of malate, succinate, and citrate oxidation
by Pi in plant mitochondria in the presence of oligomycin has
been attributed to increased substrate absorption (38). Our results
substantiate this, since Pi at concentrations often used in respira-
tory studies (5 mM) greatly stimulates citrate and succinate uptake.
Citrate uptake in the presence of Pi begins increasing at approxi-
mately 1.5 mM. This corresponds to the observation that apprecia-
table O2 uptake rates first occur at 2 to 3 mM citrate under identical
conditions but in the absence of oligomycin (Fig. 1A), suggesting
that respiration which allows energization of the mitochondria
stimulates citrate uptake. Succinate at low concentrations (0.5 mM)
is readily oxidized by potato mitochondria (not shown). The
oxidation isotherms which are sigmoid for citrate and hyperbolic
for succinate thus appear to shape the uptake isotherms.
The Km for phosphate stimulation of substrate uptake is 0.45
and 0.54 mM Pi for succinate and citrate, respectively (not shown).
A similar value was determined for the Pi stimulation of citrate
respiration in the presence of an uncoupler and oligomycin (not
shown), suggesting that respiration under these conditions is again
limited by substrate uptake. The Pi stimulation of acceptorless
oligomycin-sensitive NADH respiration and swelling in corn mitochon-
dria, processes dependent on Pi accumulation, also have Km
values in the range reported here (12). The Pi stimulation of
citrate and succinate uptake appear dependent on Pi transport.

Effect of NADH, Pi, and Malate. Exogenous NADH is readily
oxidized by intact plant mitochondria via an external dehydro-
genase linked to two coupling sites (2), with the result that the
mitochondria are energized without the need for substrate pen-
tration of the inner membrane. NADH stimulates citrate uptake

4 Abbreviations: BTCA: 1,2,3-benzenetricarboxylic acid; CCP: carbonyl
cyanide phenylhydrazone; DNP: 2,4-dinitrophenol.
TABLE 1. Effects of P_i, dicarboxylates and inhibitors on citrate uptake.

Uptake was measured at 5 min in the presence of 1 mM sodium arsenite. At 0.5 mM \( \text{[}^{14}\text{C}] \text{citrate} \) the volume was 0.6 ml with 0.25 mg protein and when added, 2.5 mM KPi and 0.5 mM K-malate. At 3 mM \( \text{[}^{14}\text{C}] \text{citrate} \) the volume was 1.5 ml with 1.03 mg protein, 5 mM KPi, and 0.25 mM K-malate. Inhibitors were added to give 10 mM BTCA, 10 mM benzylmalonate, 2 \( \mu \text{g/ml} \) antimycin, 0.1 mM DNP, and 20 and 30 nmol mersalyl/mg protein when 0.5 and 3 mM citrate were used respectively.

<table>
<thead>
<tr>
<th>Additions</th>
<th>0.5 mM Citrate</th>
<th>3 mM Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uptake (nmol/mg protein)</td>
<td>Stimulation or Inhibition (-)</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none (control)</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>P_i</td>
<td>0.8</td>
<td>-56</td>
</tr>
<tr>
<td>malonate</td>
<td>0.98</td>
<td>-51</td>
</tr>
<tr>
<td>malonate, P_i</td>
<td>0.50</td>
<td>-25</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none (control)</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>mersalyl</td>
<td>0.01</td>
<td>-85</td>
</tr>
<tr>
<td>BTCA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>benzylmalonate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>antimycin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNP</td>
<td>0.3</td>
<td>-57</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of NADH, P_i, and malate on citrate uptake isotherms.

Uptake was measured at 5 min. Reaction medium volume was 0.6 ml with the mixture containing 0.23 mg protein and when added, 1.2 \( \mu \text{mol} \) NADH, 2.5 mM KPi, and 0.5 mM K-malate.

oxidation, inhibits the P_i-stimulated uptake by 65%. NADH only partly reverses this inhibition, as would be expected if malonate was competing with succinate transport. ATP, an activator of succinic dehydrogenase in plants (27), was found to reduce uptake in the presence and absence of P_i. The presence of an ATPase inhibitor protein restricts the use of ATP as an energy source by potato mitochondria (18) whereas rat liver mitochondria can utilize ATP as an energy source to drive succinate and citrate accumulation (14, 23).

Effect of Inhibitors. Antimycin, benzylmalonate, uncouplers, and mersalyl were all found to be effective inhibitors of citrate uptake at 0.5 and 3.0 mm (Table I and Fig. 3) as well as succinate uptake at 3 mm (Table II). Succinate uptake was also blocked by malonate. Antimycin inhibits electron transport and thus substrate utilization and mitochondrial energization. The nonpenetrant anion, benzylmalonate, is a strong competitive inhibitor of dicarboxylate exchange and also inhibits tricarboxylate exchange in rat liver mitochondria (31). A related compound, 2-butylmalonate, inhibits dicarboxylate but not citrate exchange in bean mitochondria (8). Uncouplers such as DNP and CCP are thought to act by collapsing the proton gradient across the membrane, and have been shown to inhibit uptake and to cause efflux of anions (26, 32). Mersalyl, a sulfhydryl-blocking reagent, inhibits the phosphate, dicarboxylate and tricarboxylate carriers with varying effectiveness depending on the conditions (34). The mersalyl concentrations used in this study (15-30 nmol/mg protein) block only the P_i carrier (34). In bean mitochondria mersalyl inhibits the P_i and dicarboxylate carriers at 15 and 40 to 50 nmol/mg protein, respectively (8, 9).

A very selective competitive inhibitor of citrate transport in rat liver mitochondria, BTCA (31), has no significant effect on citrate uptake by potato mitochondria. By contrast, DeSantis et al. (8) found BTCA to inhibit citrate exchange in bean mitochondria. Citrate transport is readily inhibited by benzylmalonate in potato (Fig. 3).

The effect of adding inhibitors to mitochondria after a period of citrate accumulation in the presence of NADH and Pi is shown in Figure 4. Antimycin and CCP cause a rapid efflux of citrate, indicating that the maintenance of high levels of citrate in the mitochondria is energy-dependent. Benzylmalonate induces a slow efflux, while mersalyl causes a small increase in the level of citrate. Mersalyl added a few seconds before CCP was not effective in blocking the rapid CCP-induced efflux (not shown). Similar results were observed when inhibitors were added to mitochondria after succinate accumulation (not shown). The CCP-induced efflux of succinate and citrate accounts for the often observed decline in uncoupled respiration (ref. 6; Jung and Laties, unpublished).

Effect of Phosphorylating Conditions on Citrate Uptake. Citrate uptake was inhibited 75% when ADP was added initially to support phosphorylation (Table III). Under similar conditions using rat liver mitochondria Gamble (11) found a 67% inhibition. The inhibition in potato mitochondria was lowered to 19% when oligomycin was added to block oxidative phosphorylation (19). Oligomycin by itself stimulated uptake by 31%. Besides blocking ATPase activity, oligomycin also decreases the proton permeability of mitochondrial membranes (15), and an increase in the proton gradient in response to oligomycin could account for the increase in citrate accumulation. Oligomycin doubled the respiration-dependent citrate uptake by rat liver mitochondria (23). By the same token, utilization of the proton gradient in ADP phosphorylation decreases citrate uptake. The addition of ADP to initiate state 3 after a 3-min substrate state results in the loss of about 50% (3.5 nmol/mg protein) of accumulated citrate (not shown). The initiation of ATP synthesis in bean mitochondria results in extensive loss of accumulated Sr\(^{2+}\) (16).

The inhibition of citrate uptake, and decrease in citrate steady-state level evoked by phosphorylating conditions, could account for the observations of Raison et al. (35, 36) that initial suboptimal state 3 rates of respiration are overcome more effectively by state
Uptake was measured at 5 min. Reaction volume was 1.5 ml with 1.84 mg protein, 1.5 μg/ml oligomycin, 3 mM [14C]succinate and as added, 5 μmol NADH, 5 mM K-malate, 5 mM KPi, 0.5 mM ATP, 0.01 mM CCP, 21 mmol mermaryl/mg protein, 10 mM benzylmalonate and 2 μg/ml antimycin.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Succinate Uptake (nmol/mg protein)</th>
<th>% Stimulation or Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none (control)</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>11.1</td>
<td>65</td>
</tr>
<tr>
<td>NADH</td>
<td>6.0</td>
<td>32</td>
</tr>
<tr>
<td>NADH, P1</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>NADH, P1, malonate</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td>P1, malonate</td>
<td>5.8</td>
<td>-19</td>
</tr>
<tr>
<td>ATP</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>ATP, P1</td>
<td>8.3 (control)</td>
<td>-22</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none (control)</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>malonate</td>
<td>2.5</td>
<td>-74</td>
</tr>
<tr>
<td>CCP</td>
<td>1.7</td>
<td>-83</td>
</tr>
<tr>
<td>antimycin</td>
<td>2.0</td>
<td>-80</td>
</tr>
<tr>
<td>benzylmalonate</td>
<td>1.8</td>
<td>-81</td>
</tr>
<tr>
<td>mersaryl</td>
<td>0.9</td>
<td>-91</td>
</tr>
<tr>
<td>P1</td>
<td>17.3</td>
<td></td>
</tr>
</tbody>
</table>

4 than state 3 conditions. These results also indicate that the stimulatory effect of ADP on uncoupled citrate respiration in potato mitochondria (20) cannot be accounted for by increased substrate transport.

**Effect of pH on Energy-dependent Citrate Uptake.** The uptake of citrate at 3 mM in conjunction with respiration and in the presence of Pi was found to be very dependent on pH, with uptake increasing dramatically as the pH is lowered (Fig. 5). The pH response does not follow that for exchange on the tricarboxylate carrier (31) since uptake in this experiment depends on the coupling of several translocators—but does fit the anion distribution pattern for rotenone and oligomycin-inhibited rat liver mitochondria described by Palmieri et al. (29). They concluded that lowering the medium pH increased the pH difference across the inner membrane and increased anion uptake since anion distribution at equilibrium depends on the proton gradient and anion capacity of the mitochondria. Inasmuch as citrate movements are proton compensated (24) citrate uptake increases with decreasing pH. The anion capacity of rat liver mitochondria is thought to be limited by the fixed concentration of matrix cations since electroneutrality must be maintained. Respiration allows for the development of a larger anion capacity, as cation uptake may accompany anion uptake, allowing net salt accumulation. The increase in citrate uptake by potato mitochondria with decreasing pH in the presence of respiration suggests that respiration under standard reaction conditions (pH 7.5) does not provide optimal driving force for citrate uptake. Also, changes in dissociation occur as the pH decreases and increased amounts of monovalent phosphate and divalent citrate become available and this may likewise result in increased uptake.

Under the conditions of Figure 5 at pH 6.4, 9 nmol/mg protein of citrate were taken up in the presence of mersaryl and benzylmalonate. This suggests that noncarrier passive efflux also increases as the medium pH is decreased.

**Effect of Succrose on Succinate Uptake.** Optimal succinate uptake occurs at 50 mM sucrose in the absence of inhibitors, and decreases as the sucrose concentration (and osmotic pressure) is increased (Fig. 6). Because succinate oxidation influences succinate uptake in these experiments, inhibition by sucrose could be on transport, oxidation, or both. The inhibitory pattern is still observed in the presence of antimycin, however, suggesting an
effect on substrate penetration per se. Since potato mitochondria behave as osmometers, matrix volume decreases as sucrose concentration increases and this osmotic effect cannot be separated from a direct sucrose effect on the carrier as both were varied. Low uptake levels with antimycin indicate that passive influx does not become appreciable under hypotonic conditions where the relative matrix volume is large.

**DISCUSSION**

The data presented here on succinate and citrate transport are compatible with the generally accepted mechanisms of anion transport in mitochondria (10, 37). In contrast to mammalian mitochondria, freshly isolated potato mitochondria appear to be poorly energized (i.e. membrane potential and proton gradient associated with the inner membrane are not substantial) and contain low levels of endogenous exchangeable metabolites. Succinate and citrate may be taken up in mitochondria by energy-independent exchange or by energy-dependent net anion accumulation (11, 24). Figure 7 illustrates the systems postulated to be involved in these processes (10, 37). Energy-independent citrate uptake involves only exchange on the tricarboxylate carrier, whereas net citrate uptake depends on the respiratory chain and the Pi, dicarboxylate and tricarboxylate carriers. When a large proton motive force exists due to respiration or to a low medium pH, high levels of accumulation occur but when the proton motive force is small as in the presence of antimycin or uncouplers, or utilized for ADP phosphorylation, reduced mitochondrial levels result (Tables I-III and Figs. 4 and 5). Matrix substrate concentrations are thus a function of substrate oxidation and competing energy-consuming reactions.

Exchange of anions occurs in the absence of respiration and is dependent on the level of endogenous exchangeable anions. Rat liver mitochondria can exchange 10 to 15 nmol of citrate/mg protein (11, 31). Potato mitochondria exchange only 2.4 nmol of citrate/mg protein (Table I) in the presence of respiratory inhibitors, suggesting that they are relatively depleted of endogenous exchangeable anions. Rat liver mitochondria contain 25 to 40 nmol Pi and 6 to 7 nmol malate/mg protein (24, 25). We determined the Pi content of freshly isolated potato mitochondria to be 18.5 nmol/mg protein and although this does not seem to be considerably less than rat liver, the difference appears to be critical as deduced from the energy-independent uptake capacities. Compartmentation of this Pi is possibly an important factor. Potato mitochondria probably contain very low levels of malate, since in contrast to mammalian mitochondria they exhibit no endogenous respiration under the conditions used here.

Potato mitochondria resemble rat liver mitochondria depleted of endogenous metabolites by uncoupler treatment or storage (14). Depleted liver mitochondria display no endogenous respiration and lose the ability to take up citrate, which ability can be restored if a dicarboxylate and an energy source are provided (14). In the absence of respiration 3 nmol citrate/mg protein are taken up by depleted liver mitochondria as compared with 22 and 33 nmol for respiratory-inhibited undepleted liver mitochondria and succinate-restored depleted mitochondria. Respiration-inhibited potato mitochondria take up 2.4 nmol citrate/mg protein while mitochondria "restored" with NADH, malate, and Pi accumulate 17.7 nmol/mg protein (Table I and Fig. 2). The capacity of potato mitochondria to retain anions is not greatly improved by the respiratory anion-loading experiment (Table I and Fig. 4), demonstrating the need for continued energy use to maintain anions.
in the matrix and suggesting an inherent leakiness of the membranes.

In fresh rat liver mitochondria little or no increase in substrate uptake is observed with respiration in the absence of cation absorption because of the large membrane potential which develops (11). When K⁺ and an ionophore are provided to collapse the membrane potential and increase the ΔpH, anion uptake is increased severalfold. Valinomycin (40 nm) plus K⁺ (10 mM KCl) increased the uptake of 3 mM citrate in the presence of Pi by 33% in potato mitochondria, indicating a much smaller dependence on facilitated cation penetration than that found for rat liver. This disparity may be due to the greater permeability of plant mitochondria to K⁺ (13) or to an uncoupling effect of valinomycin on plant mitochondria (17). Gamble (11) found energy-dependent citrate uptake in liver mitochondria in the absence of ionophores to be much slower than energy-independent exchange, but to result in a larger accumulation, totaling 15 to 30 nmol/mg protein. Energy-dependent citrate accumulation in potato mitochondria under optimal conditions at 3 mM citrate is greater than 15 nmol/mg protein (Fig. 2).

Since the carriers operate reversibly substrate efflux may be carrier-mediated and inhibition of the carriers should slow efflux. The observations that mersalyl does not inhibit CCP-induced citrate efflux, benzylmalonate does not significantly block CCP-induced succinate efflux, and considerable efflux occurs upon benzylmalonate addition to citrate and succinate loaded mitochondria (Fig. 4, not shown), suggest that a considerable efflux occurs in potato mitochondria which is not mediated by the exchange carriers. Noncarrier passive influx appears to be limited to less than 1 nmol citrate or succinate per mg protein at 3 mM substrate as estimated from uptakes in the presence of inhibitors which should not affect noncarrier passive pathways (Table II and Fig. 4).

To study kinetics of the individual exchange carriers mitochondria must contain adequate levels of intramitochondrial anions which can exchange with externally presented substrate so the exchange itself is limiting the reaction. Since potato mitochondria contain a small pool of exchangeable anions, the extent of the citrate exchange reaction is limited, and kinetic determinations are difficult to make and cannot be derived from the experiments reported here.

Although mersalyl is very effective in blocking uptake, it actually increases citrate uptake to a small extent when added after a period of accumulation (Fig. 4), suggesting that at the level used it does not act on the tricarboxylate carrier and that high levels of citrate uptake can be maintained without the operation of the Pi carrier. This increase in citrate uptake may be due to a greater ability of citrate to compete for the available mitochondrial anion capacity in response to the inhibition of Pi uptake by mersalyl. It is not clear in these experiments if mersalyl is acting directly on the dicarboxylate carrier (Table II). BTCA does not inhibit citrate uptake, suggesting that the molecular nature of this transporter in potato mitochondria differs from other sources. BTCA was also found to be ineffective in rat heart mitochondria which demonstrate poor citrate transport ability (5). Since potato mitochondria oxidize citrate readily (18), swell in ammonium citrate in the presence of succinate and Pi (33), and have considerable capacity for citrate uptake (Fig. 2), the lack of inhibition by BTCA cannot be attributed to the lack of an active translocator.

The large oligomycin-insensitive stimulation of malate oxidation by Pi in beet root mitochondria (38) suggests that they are similar to potato mitochondria (i.e. contain low levels of exchangeable anions), whereas the small stimulation observed for corn mitochondria (6) indicates a condition more comparable to rat liver and human mitochondria. Indeed, Deyrup Husted (20) reported "passive malate absorption" of 18.5 nmol/mg protein in 3 min by antimony-blocked corn mitochondria which they assumed was not exchange-mediated. In our view this represents energy-independent malate uptake occurring by exchange with endogenous anions plus solute in extramitochondrial spaces. Nonswollen to extensively swollen heart mitochondria retain approximately 3 μl succrose-permeable water/mg protein (3). Using this value the energy-independent malate uptake by corn mitochondria from 2 mM solution calculates to about 12 nmol/mg protein. In contrast antimony-blocked potato mitochondria take up only 2 nmol succinate/mg protein in 5 min (Table II).

Isolated plant mitochondria often display depressed initial state 3 rates of respiration which rise to a maximum with several consecutive state 3/state 4 cycles. This development of state 3 respiration has been termed "conditioning" (20, 35, 36). From the data presented here, it appears that the depressed state 3 rates result largely from inadequate substrate uptake. Mitochondria become less conditioned by preincubation with inhibitors of electron transport whereas conditioning is attained by preincubation in state 4, substrate state, or a modified substrate state wherein ADP replaces Pi (35, 36). In these treatments a rise in the absolute rate of respiration accompanies the conditioning process. These observations for conditioning correlate with the citrate uptake isotherm in the presence of Pi (Fig. 1A). The later phase of the isotherm resembles the cooperative isotherm for isocitrate dehydrogenase activity and coincides with the rise of respiration which provides for net anion accumulation. Uptake reflects the citrate (hence, isocitrate) oxidation isotherm, since it is dependent on it. Transport in the absence of respiration is limited to the saturating level of uptake attained in the early part of the isotherm (Fig. 1A). This is not adequate to support rapid state 3 or uncoupler-induced respiration and hence respiration-dependent transport is necessary to achieve nonlimiting levels of substrate in the matrix. The time course of uptake should reflect the time needed for conditioning, and this is the case for the oxidation of succinate, or citrate in the presence of arsenite (Jung and Laties, unpublished).

The oxidation of succinate and citrate by unconditioned potato mitochondria is inhibited by high sucrose levels (400 mM) (ref. 36 and Jung and Laties, unpublished). Our results using freshly isolated potato mitochondria indicate that less substrate penetrates at high sucrose levels. Accordingly, at some critical level sucrose limits oxidation. Sucrose inhibition of respiration can be overcome by a conditioning pretreatment of several state 3/state 4 cycles in a 250 mM sucrose medium (4, 36). Potato mitochondria treated in

![Diagram](image-url)
this manner show no inhibition of state 3 respiration at concentrations of sucrose up to 500 mm (4). Our results suggest that substrate accumulation during pretreatment relieves the substrate transport limitation, and subsequent inhibition at concentrations greater than 500 mm sucrose may result from effects on the respiratory chain as suggested by Campbell et al. (4).

Under conditions of low substrate demand, as during the substrate state, relatively high levels of substrate are maintained in the matrix (Table III). During state 3 or uncoupled conditions when the demand for substrate becomes great, the steady-state level of matrix substrate is reduced in response to the dissipation by phosphorylation or collapse by uncouplers of the electrochemical potential across the membrane. It is this steady-state level which determines substrate limitation in the matrix. If the maintenance of this level during state 3 depended on energy utilization, it would compete with ATP synthesis for the proton motive force and undermine the phosphorylation capacity to some extent. When excess ADP is added after a 1-min substrate state the rate of state 3 respiration of potato mitochondria-oxidizing citrate does not decline but remains constant to O 2 exhaustion, indicating that adequate substrate is maintained in the matrix. The level of substrate maintained in the presence of uncouplers is quite low (Fig. 4), however, and respiration becomes inhibited due to substrate limitation.

Besides being dependent on substrate uptake, state 3 rates may also increase due to the effects of adenylates on respiratory and coupling enzyme systems (20, 27), or to the increased contribution of reducing equivalents by subsequent oxidation steps. For example, with 5 mm citrate as substrate, state 3 rates of potato mitochondria nearly double if arsenite is omitted and thiamine pyrophosphate is provided to allow oxidation of α-ketoglutarate derived from citrate oxidation (Jung and Laties, un published).

LITERATURE CITEd

4. CAMPBELL LC, JK RANKIN, CJ BRADY 1976 The response of plant mitochondria to media of high solute content. J Bioenerget Biomembr 8: 121-129
14. HARRIS EJ 1968 The dependence on dicarboxylic acids and energy of citrate accumulation in depleted rat liver mitochondria. Biochem J 100: 247-251
27. OESTRICH E, P HOYET, TP SINGER 1973 Regulation of succinate dehydrogenase in higher plants. Plant Physiol 52: 622-626
35. RANKEN JK, ML QUAGLIARIELLO, E JUNG 1973 The role of state 3 electron transport in the activation of state 3 respiration in potato mitochondria. J Bioenerget 4: 409-422
36. RANKEN JK, JM LYONS, LC CAMPBELL 1973 Inhibition of the state 3 respiration of isolated mitochondria and its implications in comparative studies. J Bioenerget 4: 397-408