Transport of Ornithine and Citrulline across the Mitochondrial Membrane*

JAMES G. GAMBLE† AND ALBERT L. LEHNINGER
From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

SUMMARY

Assay of ornithine transcarbamylase and carbamyl phosphate synthetase I in submitochondrial fractions of rat liver mitochondria show they are located primarily in the mitochondrial matrix. Therefore during the urea cycle ornithine must pass from the cytoplasm, where it is formed, to the mitochondrial matrix, where it is carbamylated. The resulting citrulline must then pass from the matrix to the cytoplasm before it can be converted to arginine. Osmotic swelling tests show that nonrespiring rat liver mitochondria do not allow the ornithine\(^+\) cation, the ornithine zwitterion, or N-acetylcitrulline to pass into the matrix, regardless of the presence of permeant anions, uncoupling agents, or valinomycin plus K\(^+\). However, when state 4 respiration is instituted with sucinate as substrate, ornithine\(^+\) readily enters the mitochondria if a permeant proton-yielding anion such as phosphate, acetate, or bicarbonate is present. However, the permeant anions nitrate and thiocyanate, which pass the membrane without carrying protons, do not support ornithine\(^+\) entry. The respiration-energized unidirectional entry of \(^{14}\)Cornithine\(^+\) is inhibited by respiratory inhibitors, by uncoupling agents, and by valinomycin plus K\(^+\), but not by oligomycin. ADP also inhibits ornithine\(^+\) entry, presumably by competing for respiratory energy. The driving force for entry of ornithine\(^+\) is concluded to be a negative-inside transmembrane potential produced when proton-conducting anions enter mitochondria to relieve the alkali-inside pH gradient generated by electron transport. It is postulated that the L-ornithine\(^+\) cation is transported by a specific electrogenic uniport carrier. This view is supported by (a) the apparent specificity of the system, which transports ornithine\(^+\) but not the closely related arginine\(^+\) or lysine\(^+\); (b) the stereospecificity for the L stereoisomer of ornithine\(^+\); and (c) the tissue specificity of ornithine\(^+\) transport, which occurs in liver mitochondria but not in those from heart, which cannot synthesize urea. The influx and efflux of citrulline in rat liver mitochondria does not depend on respiratory energy or the presence of permeant anions or cations. Because penetration of citrulline occurs into liver but not into heart mitochondria, it is postulated that citrulline also passes the membrane on a carrier, one specific for certain neutral amino acids.

The Krebs-Henseleit urea cycle is among those metabolic pathways requiring the participation of both mitochondrial and cytoplasmic enzymes. In mammalian liver the carbamylation of ornithine to citrulline, which is catalyzed by ornithine transcarbamylase, takes place exclusively in the mitochondria (1-4), as does the formation of carbamyl phosphate from ammonia, catalyzed by carbamyl phosphate synthetase I (5-7). The other steps of the urea cycle take place in the cytosol (8).

This paper reports experiments showing that both ornithine transcarbamylase and carbamyl phosphate synthetase I are located in the inner or matrix compartment of rat liver mitochondria. The characteristics of the transport processes by which ornithine enters and citrulline leaves the mitochondrial matrix have been examined. Ornithine passes through the membrane by a unidirectional process dependent upon electron transport and requiring proton-carrying anions. Citrulline passes through the membrane in either direction, without requiring respiratory energy. Our observations strongly indicate that each of these processes is promoted by a substrate-specific transport system (for reviews see References 9-11) in the inner membrane. Some of the findings described in this paper have been briefly communicated (12).

EXPERIMENTAL DETAILS

Preparation of Mitochondria and Submitochondrial Fractions—Mitochondria were isolated from livers of Sprague-Dawley albino rats by the procedure of Schneider (13) and were washed three times in cold 0.25 M sucrose. Rat heart mitochondria were prepared following Nagarse treatment (14). Protein was determined by ultraviolet absorption (15, 16); crystallized bovine serum albumin was used as standard. Mitochondria were subfractionated into an outer membrane plus soluble fraction and an inner membrane plus matrix (mitoplast) fraction by the digitonin procedure described by Schnaitman and Greenswalt (17). The mitoplast fraction was further separated into an inner membrane fraction (Lubrol-insoluble) and a matrix fraction (Lubrol-soluble), according to the method of Chan et al. (18).
Enzyme Assays—Carbamyl phosphate synthetase I (EC 2.7.25) was assayed by a modification of the procedure of Kersen and Appel (19). Bicarbonate labeled with $\mathrm{^{14}C}$ was converted in the presence of $\mathrm{NH}_3$ and $\mathrm{N}$-acetylglutamate into carbamyl phosphate, which was then reacted with citrulline in a coupled assay with excess ornithine transcarbamylase (EC 2.1.3.3). The resulting acid-stable, nonvolatile radioactivity was determined by liquid scintillation counting. The assay was linear with time and protein concentration under the conditions employed. Glutamate dehydrogenase (EC 1.4.1.2) was assayed according to Beaumay et al. (20), malate dehydrogenase (EC 1.1.1.37) and monoamine oxidase (EC 1.4.3.4) according to Schmittman et al. (21), and ornithine transcarbamylase by the method of Schimke (22). All membranous fractions were treated with 0.2 mg of Lubrol WX per mg of mitochondrial protein prior to enzyme assays.

Osmotic Swelling—The general method of Chappell and Crofts (23) was employed. Mitochondrial volume changes were observed by recording the change in absorbance of mitochondrial suspensions at 700 nm and 25°C in isosmotic (250 mosm) solutions of various solutes, supplemented with 1.0 mM Tris buffer (pH 7.4), 0.5 mM EGTA$^1$ to suppress changes due to movements of endogenous Ca$^{2+}$ and Mg$^{2+}$, and rotenone (0.5 mM) to suppress endogenous respiration. Swelling due to entry of solute and the accompanying water results in an increase in the light transmitted, which was recorded on strip charts with a Gilford-Beckman DU spectrophotometer. The various ornithine salts used in these experiments were prepared by ion exchange on DEAE-Sephadex A-25 anion exchanger (Pharmacia Chemicals, Piscataway, N. J.).

Uptake of $[^{14}C]$Ornithine—Mitochondria were recovered from the test medium on 0.45 μ Millipore filters. The filters were immediately dried under a lamp and counted in a toluene-based scintillation fluid. Results obtained by the filtration procedure were in good agreement with those obtained by centrifugation of the suspension followed by counting of the supernatant medium and pellet.

Reagents—D- and l-Ornithine, N-acetylornithine, L-lysine, and lactic dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo., malic dehydrogenase from Worthington Biochemicals, Freehold, N. J., sodium $[^{14}C]$bicarbonate (11.4 mCi per mmole) from New England Nuclear, Boston, Mass., and $[^{14}C]$ornithine (87 mCi per mmole) from Amersham-Searle, Arlington Heights, Illinois.

RESULTS

Intramitochondrial Location of Ornithine Transcarbamylase and Carbamyl Phosphate Synthetase I—Although these enzymes have long been known to reside exclusively in the mitochondria of liver tissue (1–8), their specific intramitochondrial location has not been reported. Data in Fig. 1 show their distribution in subfractions of rat liver mitochondria, compared with the distribution of the marker enzymes malate dehydrogenase (matrix), monoamine oxidase (outer membrane), and cytochrome oxidase (inner membrane). Both ornithine transcarbamylase and carbamyl phosphate synthetase I (see Reference 8) are primarily localized in the matrix, since their distribution follows closely that of the matrix markers glutamate and malate dehydrogenases.

$^1$ The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; NEM, N-ethylmaleimide.

From these data it is clear that in the operation of the urea cycle ornithine generated by the arginase reaction in the cytosol must pass through the outer and inner mitochondrial membranes into the matrix before it can undergo transcarbamylation from carbamyl phosphate formed in the mitochondrial matrix by carbamyl phosphate synthetase I. The resulting citrulline must then pass from the mitochondrial matrix through the two mitochondrial membranes to the cytosol, where the subsequent steps of the urea cycle take place. Throughout the remainder of this paper the reasonable assumption is made that the outer membrane is permeable to both these metabolites, as is true for most small solute molecules, and that the inner membrane is the true permeability barrier (9, 10).

Nonpenetration of Respiration-inhibited Mitochondria by Ornithine Salts—Since ornithine has an isoelectric pH of about 9.7, it is largely present as a cation at pH 7.4. Therefore, net movement of ornithine$^+$ into the matrix compartment of nonrespiring mitochondria can occur only if electroneutrality of the latter can be maintained. The three sets of circumstances which would meet this condition are (a) if a permeant anion enters the matrix with ornithine$^+$ in stoichiometric symport, (b) if the ornithine$^+$ cation exchanges with some matrix cation, such as H$^+$ or K$^+$, in stoichiometric antipart (c) if ornithine$^+$ is first converted enzymatically into an electroneutral species. To test these possible modes of entry rat liver mitochondria were suspended in isosmotic solutions of ornithine salts (250 mosm) containing rotenone to inhibit endogenous respiration and the requirements for osmotic swelling due to entry of ornithine examined. It was found that respiration-inhibited rat liver mitochondria completely failed to swell when suspended in isosmotic solutions of ornithine chloride (anion impermeant) or ornithine nitrate, acetate, or phosphate (anions permeant) (Table I). Thus the ornithine$^+$ cation fails to enter respiration-inhibited mitochondria, resembling in this respect the failure of K$^+$ and Na$^+$ to penetrate the membrane of nonrespiring rat liver mitochondria in the absence of ionophores (23). The failure of the mitochondria to swell in an ornithine acetate medium (Table I) further indicates that the neutral zwitterionic...
species of ornithine does not enter mitochondria, in contrast to the neutral NH₄ molecule, which can enter readily (23). Mitochondria suspended in 250 mm N-acetylmorphine also failed to swell, an observation which excludes the possibility that the ornithine⁺ cation is first acetylated to an electrically neutral species by an enzymatic reaction in the cytosol to facilitate its transport.

The impermeability of respiration-inhibited mitochondria to ornithine salts is most strikingly shown by the experiment in Fig. 2, which demonstrates that the absorbance of suspensions of rat liver mitochondria is a linear function of the osmolarity of ornithine chloride in the range of 50 to 250 mosm, in a relationship identical with that given in media of KCl, which has been earlier established to be impermeant (23). Since the absorbance is inversely related to mitochondrial volume under such conditions (28), it is clear that the rat liver mitochondria behave as nearly perfect osmometers when suspended in ornithine chloride solutions.

Addition of the ionophores gramicidin or valinomycin to respiration-inhibited mitochondria suspended in isosmotic ornithine media failed to evoke swelling (Table 1), whether the anion was impermeant (chloride) or permeant (nitrate). Moreover, no swelling was observed in such systems adjusted to pH 8.3, at which the membrane is permeable to chloride and other anions (24-26). In appropriate control experiments addition of the ionophores to mitochondria suspended in KCl at pH 8.3 or in KNO₃ (27) evoked immediate swelling. It was concluded that neither gramicidin nor valinomycin promote the entry of the ornithine cation.

When the proton-conducting uncoupling agent FCCP was added to respiration-inhibited mitochondria suspended in isosmotic ornithine salts, no swelling took place (Table 1). In control experiments, addition of FCCP did cause swelling of mitochondria suspended in NaNO₃ (27) in confirmation of earlier conclusions (28) that rat liver mitochondria contain a Na⁺-H⁺ exchange or antiport system, whose activity can be observed in the presence of FCCP to allow external protons to enter mitochondria to replace internal protons exchanged for external Na⁺. The failure of FCCP to stimulate swelling in ornithine nitrate thus indicates that rat liver mitochondria do not contain a system which promotes ornithine⁺-H⁺ antiport.

Penetration of Ornithine⁺ into Respiring Mitochondria—The effect of induction of respiration on ornithine entry was then examined. When succinate was added to mitochondria suspended in ornithine chloride, no significant swelling ensued. However, when succinate was added to mitochondria suspended in isosmotic ornithine media containing the anions acetate or phosphate, rapid swelling of the mitochondria took place (Fig. 3).

Not all permeant anions will support respiration-dependent entry of ornithine⁺. Although phosphate and acetate support
ornithine$^+$ entry, the permeant anions NO$_3^\text{-}$ and SCN$^\text{-}$ do not (Table I). This observation is of considerable importance (see "Discussion"), since phosphate and acetate are transported across the membrane as the protonated species H$_3$PO$_4^\text{-}$ and HOAc, respectively, whereas the nitrate and thiocyanate anions pass the membrane as such (23). As will be seen below, bicarbonate also can support entry of ornithine$^+$. However, induction of respiration by succinate failed to cause N-acetylornithine to enter.

The experiments in Fig. 3 also show that the respiratory inhibitors antimony A and cyanide inhibit succinate-induced swelling in ornithine phosphate medium. The uncoupling agent FCCP also completely prevented respiration-induced swelling in ornithine acetate. Antimycin had no effect, indicating that the coupled formation of ATP was not a requirement for swelling. Respiratory substrates other than succinate also supported ornithine$^+$ uptake, as will be shown below. Moreover, endogenous respiration of rat liver mitochondria, which is NAD dependent, can also support swelling in ornithine phosphate media when rotenone is omitted.

Direct gravimetric measurements showed that the decrease in light absorption of respiring mitochondria suspended in ornithine phosphate is actually due to uptake of water. Increases in mitochondrial volume up to 2.5-fold were observed before apparent lysis occurred.

**Participation of Phosphate Carrier in Respiration-dependent Penetration of Ornithine Phosphate**—Phosphate enters the matrix compartment of rat liver mitochondria in a specific carrier-mediated process, probably through H$_2$PO$_4^\text{-}\text{--OH}^\text{-}$ antiport (23). This carrier is inhibited by mersalyl (29-31), p-chloromercuribenzoate (32), formaldehyde (33), and NEM (32). Table I shows data on the effect of NEM on the succinate-dependent swelling of rat liver mitochondria in ornithine phosphate and ornithine acetate media. NEM blocked swelling in ornithine phosphate but had no inhibitory effect on energized swelling in the ornithine acetate medium. These results therefore show that entry of ornithine$^+$ into respiring mitochondria requires entry of the phosphate anion via the NEM-sensitive phosphate carrier. However, the entry of acetate, which is believed to enter by simple nonmediated diffusion of free acetic acid, is insensitive to NEM.

**Isotopic Measurements of Entry of Ornithine$^+$**—Data in Table II show that $^{14}$C-labeled ornithine$^+$ enters rat liver mitochondria when they are supplemented with succinate and phosphate. Omission of either succinate or phosphate reduced the uptake of isotope to very low levels. Uptake of isotope was also completely inhibited by FCCP, antimycin A, and cyanide, but not by oligomycin. The results obtained with radioactive ornithine thus fully agree with those observed with the osmotic swelling method.

Data in Table II also show that valinomycin in the presence of K$^+$ completely inhibits ornithine uptake, in agreement with
many observations that this ionophore imposes a drain on respiratory energy, making it unavailable for oxidative phosphorylation and other respiration-dependent activities of mitochondria.

Succinate, β-hydroxbutyrate, and pyruvate plus malate were about equally effective as substrates in supporting ornithine uptake; α-ketoglutarate was somewhat less effective (Table II). Glutamate was significantly more active than other substrates. This observation may be accounted for by the fact that conversion of ornithine to citrulline in the mitochondrial matrix required, in addition to ATP, sources of ammonia and bicarbonate, both of which can be supplied by oxidative degradation of glutamate. However, addition of ammonium chloride to the system did not stimulate succinate-supported ornithine uptake.

It is also seen (Table II) that the permeant anions bicarbonate and acetate can replace phosphate in supporting ornithine uptake; however, the permeant anions nitrate and thiocyanate are relatively inactive, in agreement with the osmotic swelling data shown above and with earlier experiments on support of respiration-dependent Ca\(^{2+}\) uptake by proton-yielding anions (34).

**Rate and Extent of Ornithine Uptake**—Fig. 4 shows that the energized uptake of \[^{14}C\]ornithine\(^{+}\) is linear with time up to 20 min at 37°C. The observed rate of uptake, starting from 5 mM ornithine\(^{+}\), was 2.9 nmoles per min per mg of protein. In other experiments rates exceeding 5 nmoles per min per mg of protein were observed. Although this is relatively low compared to the rate of energy-linked Ca\(^{2+}\) uptake, which may exceed 350 nmoles of Ca\(^{2+}\) per mg per min under similar conditions, it is of the same order of magnitude as the rate of urea synthesis in rat liver (see "Discussion").

In the experiment of Fig. 4 about 60 nmoles of ornithine per mg of protein were accumulated, but in other experiments uptakes exceeding 160 nmoles of ornithine per mg of protein were observed. If it is assumed that the volume of the matrix water after maximum ornithine uptake is 2.0 μl per mg of protein, the internal concentration of ornithine achieved in the matrix water in such experiments may exceed 80 mM, some 16-fold higher than the external concentration of 5 mM. In similar experiments it was found that when the external ornithine concentration was initially at 5 μM, internal to external ornithine ratios greatly exceeded 200:1. These calculations assume that all the radioactive recovered in the mitochondria is in the form of ornithine.

The initial rate of ornithine entry at low initial concentrations of ornithine is difficult to measure precisely because of the relatively long times required to filter or centrifuge the mitochondria in relation to the initial rate of uptake. However, data in Fig. 5 suggest that the initial rate of entry of ornithine\(^{+}\) in the first minute approaches a saturation level as the ornithine concentration is increased. The concentration of ornithine\(^{+}\) yielding approximately half-maximal initial rates of isotope uptake appears to be less than 500 μM, which may be compared with the concentration of ornithine in the cell water of rat liver, estimated to be in the range of 133 to 484 μM (35). On increasing the external ornithine concentration to much higher levels (5 to 10 mM) and the incubation time to 15 to 20 min, the saturation effect was lost. Under the latter conditions, which lead to extensive swelling of the mitochondria, some of the ornithine was evidently entering by unmediated concentration-dependent diffusion; presumably stretching of the membrane leads to a generalized increase in its permeability.

**Effect of ADP and Ca\(^{2+}\) on Uptake of Ornithine**—Data in Table II show that respiration-dependent \[^{14}C\]ornithine uptake supported by oxidation of succinate is severely inhibited by ADP. Approximately half-maximal inhibition was given by as little as 0.2 mM ADP. Ca\(^{2+}\) (1.0 mM) inhibited ornithine uptake nearly completely. These data indicate that phosphorylation of ADP and energy-requiring accumulation of Ca\(^{2+}\) take precedence over ornithine transport for energy generated by electron transport. However, other factors may be involved in the effect of ADP on ornithine\(^{+}\) uptake (see "Discussion").

**Effect of Ornithine\(^{+}\) on Oxygen Uptake and H\(^{+}\) Ejection**—Addition of ornithine\(^{+}\) in high concentrations (1 to 10 mM) yielded no detectable stimulation of the state 4 rate of oxygen uptake of rat liver mitochondria respiring on succinate, in comparison with the large stimulation yielded by ADP or Ca\(^{2+}\). This finding is consistent with the relatively low rate of ornithine uptake as compared to the rate of oxidative phosphorylation or Ca\(^{2+}\) accumulation. Addition of 10 mM ornithine to state 4 moto-

![Graph](http://example.com/graph1.png)
Table III

Osmotic swelling of mitochondria in isosmolar citrulline and other amino acids

The test media contained 250 mosM amino acids, 1.25 mM rotenone to suppress endogenous respiration, 0.5 mM EGTA, mitochondria (1 mg of protein per ml), and other additions as shown. The temperature was 25°.

<table>
<thead>
<tr>
<th>Source of mitochondria</th>
<th>Amino acid</th>
<th>Rate of swelling (ΔAue/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>Ornithine chloride</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Citrulline</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>Citrulline + succinate</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>Citrulline + FCCP</td>
<td>0.085</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>0.350</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>0.380</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>0.410</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>0.500</td>
</tr>
<tr>
<td>Experiment 2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>Citrulline</td>
<td>0.120</td>
</tr>
<tr>
<td>Rat heart</td>
<td>Citrulline</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Mitochondria also yielded little or no ejection of H⁺ into the medium, in contrast to the large and rapid ejection of H⁺ given by Ca²⁺. The observations reported here show that the membrane of respiring blowfly muscle mitochondria and the observations of Hansford and Lehninger (38) on entry of K⁺ into the matrix of beef heart mitochondria, and the observations of T. O. L. Hansford and A. Lehninger (38) on entry of K⁺ into the matrix of beef heart mitochondria. In both cases, phosphate, acetate, and bicarbonate, have as common denominator the ability to yield protons when they enter into the matrix.

Influx and Efflux of Citrulline—Experiments in Table III show that mitochondria swell in the presence of citrulline, which is predominantly in the electrically neutral zwitterion species at pH 7.0, without a requirement for either respiratory energy or phosphate. The rate of swelling in isotonic citrulline media was unaffected by either permeant (acetate, thiocyanate, or phosphate) or impermeant (chloride) anions. Moreover, the entry of citrulline was not influenced by respiratory inhibitors such as cyanide or rotenone or by uncoupling agents such as FCCP. Other neutral (zwitterionic) amino acids (proline, valine, alanine, and glycine) also enter mitochondria in a similar passive manner under these conditions (Table III). The rate of citrulline entry is relatively modest compared to that of glycine, which is the most active. Rat liver mitochondria preloaded with [14C]citrulline and then added to an isotonic sucrose medium were found to undergo a rapid loss of isotope to the medium (Fig. 6), showing that citrulline can readily pass through the mitochondrial membrane in either direction. On the other hand, rat liver mitochondria preloaded with [14C]ornithine did not lose isotope under these conditions.

Data in Table III show that rat heart mitochondria, which cannot form citrulline from ornithine, do not swell when suspended in an isosmotic citrulline medium. The tissue specificity suggests the possibility that citrulline transport is also mediated by a carrier in rat liver mitochondria.

Discussion

This exploratory investigation of the transport of ornithine and citrulline across the mitochondrial membrane provides information regarding (a) the driving force for transport (b) the mechanism of transport, and (c) the metabolic organization of the urea cycle in the liver.

The observations reported here show that the membrane of respiring-inhibited rat liver mitochondria is impermeant to ornithine, whether as the cation, the electrically neutral zwitterion, or as N-acetylornithine. However, the ornithine⁺ cation readily enters the mitochondrial matrix when electron transport is taking place, in the presence of certain permeant anions. The permeant anions promoting entry of ornithine⁺, which include phosphate, acetate, and bicarbonate, have as common denominator the ability to yield protons when they enter into the matrix. Acetate is believed to cross the membrane as the undissociated free acid (23), phosphate is transported as either HPO₄²⁻ or H₂PO₄⁻, most likely the latter (23, 27), and bicarbonate crosses the membrane as dissolved CO₂; the dissolved CO₂ presumably is rehydrated in the matrix to yield the proton-bearing bicarbonate ion (34). On the other hand, ornithine⁺ entry is not supported by the permeant anions SCN⁻ and NO₃⁻, which cross the membrane as such and do not occur to any significant extent as protonated species at pH 7.4.

These observations on the entry of the ornithine⁺ cation closely resemble the observations of Drierley and his colleagues (36, 37) on the respiration-dependent entry of K⁺ and other cations into the matrix of beef heart mitochondria and the observations of Hansford and Lehninger (38) on entry of K⁺ into the matrix of respiring blowfly muscle mitochondria. In both cases, phosphate or the anions of weak acids were required for respiration-dependent entry of K⁺. Moreover, the anion requirements for
ornithine$^+$ entry are also identical with those established for the respiration-dependent entry of Ca$^{2+}$ into rat liver mitochondria, which is supported by phosphate, acetate, and bicarbonate, but not by nitrate or thiocyanate (34, 39). Thus it appears that the driving force for entry of the ornithine$^+$ cation is the same as that involved in the respiration-dependent entry of K$^+$ and Ca$^{2+}$. The requirement of a proton-yielding anion is accounted for by the generation of an alkaline-inside proton gradient by electron transport, for which the model of Mitchell (40) provides the simplest but not the only possible rationale. The alkaline-inside proton gradient pulls in the proton-yielding anion in the matrix. The negative-inside potential gradient developed in this manner is responsible for electrophoretic transport of the cation into the matrix, whether it is ornithine$^+$, K$^+$, or Ca$^{2+}$ (Fig. 7). Arguments supporting such a role of phosphate and the anions of weak acids have been developed in other communications from this laboratory (34, 39), and by Brierley and his colleagues (36, 37).

We may now examine the nature of the process by which the ornithine$^+$ cation passes through the membrane of rat liver mitochondria in response to the electrophoretic driving force. Our swelling test results show that the $\alpha$ stereoisomer of ornithine enters but the $\beta$ isomer does not; in addition it was shown that the structurally similar amino acids L-lysine and L-arginine do not enter. Moreover, it was found that ornithine enters respiring rat liver mitochondria, which participate in urea synthesis, but not heart mitochondria, which do not. Moreover, ornithine entry occurs by a process that appears to approach saturation at relatively low concentrations. All these properties strongly suggest that the ornithine$^+$ cation enters rat liver mitochondria in response to an electrophoretic driving force via a specific carrier or transport system rather than by simple unmediated physical diffusion through the membrane, which would be expected to be nonspecific for the stereoisomers and to be concentration-dependent.

The hypothesis of an ornithine$^+$-specific carrier or transport system functioning in response to an electrochemical gradient generated by electron transport is not, however, sufficient to explain all our findings. We have found that nonrespiring rat liver mitochondria do not swell when placed in isosmolar ornithine nitrate or thiocyanate, conditions in which both the ornithine$^+$ cation and the permeant anion would be expected to move down rather steep electrochemical gradients into the matrix if the membrane contained an ornithine-specific carrier. Nor do they swell in media of ornithine phosphate or acetate, even in the presence of FCCP to allow protons brought in with phosphate or acetate to escape from the matrix. Only after institution of respiration in the presence of proton-yielding anions does ornithine enter, whether from concentrated (125 mM) or dilute (50 mM) media. Again, these findings are similar to those on entry of K$^+$, which readily passes through the membrane of heart (36, 37) and blowfly (38) mitochondria when they are respiring in state 4 but do not pass the membrane of respiration-inhibited mitochondria unless an ionophore such as gramicidin or valinomycin is present. To explain the requirement of respiration for ornithine$^+$ entry we postulate that in nonenergized, respiring mitochondria the ornithine carrier is in an inactive form whereas during state 4 respiration it is in an active state and can then transport ornithine$^+$ inward electrophysically in response to the negative-inside potential generated by electron transfer. This proposal (see also Reference 37) is consistent with much evidence that the mitochondrial membrane undergoes very significant microscopic and molecular changes in its conformation during transitions between state 4 and state 3, as detected with the electron microscope (41) and with fluorescent probe methods (42).

The experiments reported here suggest that citrulline enters and leaves rat liver mitochondria passively by a respiration-independent process. However, since rat liver mitochondria undergo rapid swelling in isosmolar citrulline media, but heart mitochondria do not, it appears likely that rat liver mitochondria contain a carrier capable of transporting citrulline as well as other neutral amino acids (cf. Reference 43). Tests of the stereospecificity of citrulline transport should furnish significant evidence on this point. We have considered the possibility that the entry of the ornithine cation and the exit of the citrulline zwitterion may take place by an obligatory antiport process, in keeping with the fact that during urea synthesis one molecule...
Whether the remaining urea cycle intermediate, argininosuccinate, is established. The question arises whether the transport of ornithine is responsible for the fluctuations from one preparation of mitochondrion to another in rate and extent of the uptake of isotope from the [14C]ornithine media.

Fig. 9. Summary of role of electron transport-generated energy in mechanism of urea synthesis. TCA cycle, tricarboxylic acid cycle.

Aspartate and ammonia for the synthesis of carbamyl phosphate, with the requirement for the availability in the matrix of ATP, bicarbonate, and ammonia for the synthesis of carbamyl phosphate, with the requirement for the formation of argininosuccinate from aspartate generated by the tricarboxylic acid cycle and ammonia formed by oxidative deamination of amino acids. Secondly, the mitochondrion also generates one molecule of external ATP required for the formation of argininosuccinate from aspartate and citrulline in the cytoplasm. Thirdly, the mitochondrion may also yield an extra thermodynamic “push” in the direction of urea synthesis by bringing about unidirectional transport of ornithine into the matrix at the expense of respiratory energy (Fig. 9).

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