Kinetics and Regulation of the Glutamate-Aspartate Translocator in Rat Liver Mitochondria*

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The kinetics and mechanism of the electrogenic exchange of external glutamate with intramitochondrial aspartate have been investigated using aspartate-loaded rat liver mitochondria in the absence of metabolism. Apparent kinetic constants were calculated from measured decreases of the mitochondrial matrix aspartate content after glutamate addition by a computer curve-fitting procedure and by graphical analysis. An increase of external glutamate concentration caused an increase of $V_{\text{max}}$, but only small changes of the $K_{s}^{-}$, for matrix aspartate. The decrease of the $K_{s}^{-}/V_{\text{max}}$ ratio with increasing glutamate concentration rules out a ping-pong mechanism for the translocator, and indicates a ternary complex between the carrier, glutamate, and aspartate.

The dependencies of aspartate efflux kinetics on the mitochondrial membrane potential ($\Delta\Psi$) and $\Delta p$H were also investigated in aspartate-loaded mitochondria. The $V_{\text{max}}$ of aspartate efflux was not affected by changes of the external pH over the range from 6.5 to 7.8 or by changes of the internal pH over the range of 6.8 to 9.0. However, the $V_{\text{max}}$ of aspartate efflux was linearly dependent on $\Delta\Psi$ over the range from 70 to 160 mV. Under all conditions investigated, including the de-energized state, the $K_{s}^{-}$ of the glutamate-aspartate translocator for matrix aspartate remained approximately constant. These data indicate (a) binding of uncharged glutamate to the carrier, and (b) that the translocation of a negatively charged carrier-aspartate complex across the mitochondrial membrane is the rate-limiting step of the reaction sequence.

Glutamate-independent kinetic constants were calculated from the apparent kinetic constants obtained from the computer and graphical fitting procedures. Average values were 3.9 nmol/mg for the matrix aspartate $K_{s}^{-}$, 5.8 mm for the external glutamate $K_{s}^{-}$, and 21.2 nmol/mg/min for the $V_{\text{max}}$ at 10°C. Using these constants, together with a $K$ for external aspartate of 4 mm, and a temperature coefficient of 15.1 kcal/mol, the aspartate translocator kinetics were described in terms of a rate equation. Values of mitochondrial aspartate efflux in isolated hepatocytes calculated using this rate equation were in approximate agreement with measured rates. It is concluded that no significant microcompartmentation of aspartate occurs in the mitochondrial matrix in the intact cell.

Previous studies (for review, see Ref. 1) have shown that the mitochondrial glutamate-aspartate translocator may be classified along with the adenine nucleotide translocator as electrogenic, since exchange of anions in the transport process is associated with a charge imbalance across the mitochondrial membrane. During transport in the physiological direction, the adenine nucleotide translocator catalyses an exchange of extramitochondrial ADP$^{-}\cdot$ with intramitochondrial ATP$^{-}$ (2) while, with the glutamate-aspartate translocator, external glutamate is transported on the carrier as the uncharged species in exchange with the monovalent aspartate anion (3). Glutamate may also be transported across the mitochondrial membrane by a separate electroneutral Glu/OH antiporter mechanism (4, 5), but the glutamate-aspartate translocator is presently the only known mechanism for aspartate transport. The electrogenic properties of the glutamate-aspartate translocator have important consequences for cell function. First, it is energy dependent, since the net efflux of negative charge will collapse a previously established membrane potential. Continued transport of aspartate thus requires expenditure of energy by the electron transport chain or by extramitochondrial ATP to maintain the proton electrochemical gradient (6, 7). Second, aspartate flux is asymmetrical in the energized state since entry of the uncompensated charge of the aspartate anion into the mitochondria is impeded by the membrane potential (negative inside). Thus, the rate of aspartate entry into mitochondria in exchange with glutamate is appreciable only when the membrane potential is collapsed. Under these conditions, aspartate efflux is inhibited (3, 7). Third, the rate of mitochondrial aspartate transport under physiological conditions in the cell is expected to be determined by the kinetic properties of the translocator. Thus, the ratio of the glutamate to aspartate gradients on either side of the mitochondrial membrane is far displaced from equilibrium with the membrane potential (1), hence aspartate efflux is more likely to be regulated by kinetic than energetic considerations. A rather precise regulation of the glutamate-aspartate translocator is required in the cell since aspartate efflux from the mitochondria is an obligatory step in the transport of reducing equivalents into mitochondria by the malate-aspartate cycle (6), in gluconeogenesis from lactate (8), and in urea synthesis (9). Previous studies have shown that external aspartate inhibits noncompetitive inhibition with glutamate ($K_{s}^{-}$ of 6 to 7 mm) with a $K_{s}^{-}$ of about 4 mm (3). However, the kinetic constants on the matrix side of the inner mitochondrial membrane are not known.

In the present paper, aspartate depletion from the mitochondrial matrix has been measured as a function of time.
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Aspartate Efflux—Several methods may be used for kinetic analysis of mitochondrial aspartate depletion curves such as those illustrated in Fig. 1. One method is to regard the transport process as first order with respect to aspartate and express the kinetics in terms of first order rate constants (18). Half-times calculated from the semilog plots should behave in the same way as the $K'_m/V_{m,18}$ ratio. The major disadvantage of this method is that it disregards much of the information implicit in the curves and implies lack of saturation kinetics characteristic of transport processes. An alternative method, which is preferable to the above because it accounts for saturation but assumes no specific mechanism, is expression of the data as initial rates of aspartate efflux. However, a description more compatible with the experimental measurements of the transport kinetics is afforded by use of the integrated rate equation given below.

The experimental conditions for measurement of the translocation kinetics are: (a) extramitochondrial glutamate concentration does not change significantly during a time course; (b) extramitochondrial aspartate concentration remains insignificant relative to its known translocator inhibition constant (3); (c) extramitochondrial aspartate does not interact with the carrier to affect significantly the binding of intramitochondrial aspartate; and (d) at equilibrium, some matrix aspartate remains. Thus, a reversible Uni-Uni enzyme rate equation with apparent, steady state $K_m$ and $V_{m,18}$ values will operationally describe aspartate-glutamate kinetics for the reaction scheme:

$$E + S = ES = EP = E + P$$

The extremely small ratio of extramitochondrial to matrix aspartate under the experimental conditions makes it unnecessary to consider two forms of the carrier, as depicted for the iso Uni- Uni mobile carrier model of membrane transport described by Segel (19). It is implied in the use of the Uni-Uni rate equation that there is no inhibitor or activational effect by matrix glutamate on the transport rate (3). That the simple equation describes aspartate efflux time courses within experimental error is strong evidence for this assumption at least in the experimental concentration ranges.

The Uni-Uni steady state rate equation derived by Hamme and Albert (20) which suitably describes the glutamate-aspartate translocator is:

$$-\frac{dS}{dt} = \frac{(V_m/K_m)S - (V_m/K_m)P}{1 + S/K_m + P/K_m}$$

where $S$ and $P$ are the matrix and external aspartate concentrations, respectively. The superscripts with maximum rate ($V_{m,18}$) and Michaelis constant ($K_m$) refer to efflux (r) and influx (i) aspartate translocation directions. These steady state parameters are dependent upon extramitochondrial glutamate concentration, and their interpretation depends upon the postulated translocation scheme.

Since the kinetic observations of the glutamate aspartate translocation are in the form of matrix aspartate time courses, Equation 1, which describes the data, must be integrated with respect to time (i) and matrix aspartate $S$. Application of conservation of mass for $S$ and $P$ and operational definitions of maximum rate and Michaelis constant gives an integratable form of the equation:

$$-\frac{dS}{dt} = \frac{(V_m/K_m)S - (V_m/K_m)P}{K_m + S}$$

where $V_{m,18} = (V_{m,18}/K_m + V_{m,18}/K_m)$ and $K_m = (1 + S/K_m + P/K_m)$. The abbreviations used are: Mops, 3-N-morpholinopropanesulfonic acid; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; DMO, 5,5'-dimethyloxazolidine-2,4-dione; DMO, 5,5'-dimethyloxazolidine-2,4-dione; and DPO, 5,5'-dimethyloxazolidine-2,4-dione.
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$S_m$ is the aspartate conservation condition ($S + P$), and $S_m$ is the final concentration of matrix aspartate (i.e., when $dS/dt = 0$). For highly reversible cases, the $K'$ dependence upon $S_m$ demands that $S_m$ be kept constant for comparable experimental series; in the case at hand, the translocation typically progressed to more than 90% completion, leaving $S_m/K'_m$ buried in experimental error (i.e., $K'_m \approx K'_m$). The implicit time course of matrix aspartate, from the integration of Equation 2, is:

$$V_{max} - (K'_m + S_m) \ln \frac{S_m - S_{in}}{S - S_{in}} - (S_0 - S) = 0$$  \hspace{1cm} (3)

where $S_0$ is the initial matrix aspartate concentration (equal to $S_m$ in these experiments). The low degree of reversibility of the glutamate aspartate translocator under the given experimental conditions is consistent with:

$$V' \approx V_{max} \text{ and } K'_m \approx K'_m$$

Although Equation 3 is derived for matrix aspartate, appropriate use of the conservation condition $S_m$ will convert the equation to a description of the external aspartate time course.

Equation 3 can be resolved either by computer curve fitting procedures, presented elsewhere (21), or by a graphical method (22). The graphical method is based on an algebraic rearrangement of Equation 3 and the useful definitions:

$$s = \frac{S_m - S}{\ln(S_0 - S_m)/(S - S_m)} \text{ (units of substrate)}$$

$$v = \frac{S_m - S}{t} \text{ (units of rate)}$$

The substitution of these definitions into Equation 3, followed by solution for $v$, yields:

$$v = \frac{V_{max} s}{(K'_m + S_m) + s}$$  \hspace{1cm} (4)

Although $s$ and $v$ are not rate and concentration, respectively, in the usual sense, Equation 4 is formally the Michaelis-Menten equation; thus, the same linearization techniques commonly applied to the Michaelis-Menten equation also apply to Equation 4. For this investigation, the Lineweaver-Burk transformation is used, i.e.:

$$1/v = [(K'_m + S_m)/V_{max}]1/s + 1/V_{max}$$  \hspace{1cm} (5)

Equation 5, which implicitly describes the time course for a reversible Uni-Uni, reaction, has the same $1/v$ versus $1/s$ slope and intercept concentration dependencies as the equivalent initial rate Lineweaver-Burk graphs. Furthermore, changes in the slopes or intercepts for a series of experiments, or both, can be interpreted in the manner of Cleland (23). For example, a series of lines with increasing external glutamate concentrations should show decreasing slopes and intercepts with a simultaneous intersection in the second or third graph quadrant if the translocation proceeds via a kinetically significant ternary complex of translocator, aspartate, and glutamate.

Materials—Aminooxyacetate (carboxymethoxyamine hemihydrochloride) was obtained from Aldrich Chemical Co. TMPD was purchased from Eastman Organic Chemicals, and rotenone from K and K Laboratories. Mannitol for mitochondrial preparations was purchased from Baker Chemical Company because of its low calcium content. Enzymes were obtained from Boehringer Mannheim Corporation and radioactive isotopes from New England Nuclear. Nigericin was a gift from Eli Lilly and Company. All other chemicals were obtained from Sigma Chemical Company.

RESULTS

Effect of Glutamate on Aspartate Efflux—Time courses of aspartate efflux from aspartate-loaded mitochondria at a number of different medium glutamate concentrations from 1.5 mM to 15 mM are shown in Fig. 1. The mitochondria were incubated at 10°C in KCl medium, pH 7.2. The data points shown are averages calculated from changes of aspartate in both the mitochondrial matrix and the medium. The solid lines represent the computer fitted curves for each data set.

Using the graphical method of analysis (Equation 5) to linearize the integrated rate equation description of transport kinetics (Equation 3), double reciprocal plots of the data shown in Fig. 1 give a series of converging straight lines that intersect close to the abscissa (Fig. 2). A comparison of apparent $V_{max}$ and $K'_m$ values calculated from the computer curve fitting procedure (21) and the double reciprocal graphical method is shown in Table I. Both methods give similar values for both $V_{max}$ and $K'_m$ it is seen that as the medium glutamate concentration increases with a constant initial intramitochondrial aspartate content, the $K'_m$ remains approximately constant while the $V_{max}$ increases.

The $K_m$ for external glutamate and the glutamate concentration independent $K_m$ and $V_{max}$ values for aspartate efflux can be calculated using the conventional Equations 6 and 7, as for initial rate analyses:

$$1/V_{max} = 1/V_{max} + (K'_m/V_{max}/G_0$$  \hspace{1cm} (6)

$$K'_m/V_{max} = K'_m/V_{max} + K'/V_{max}/G_0$$  \hspace{1cm} (7)

where $K'_m$ is the matrix aspartate Michaelis constant (nmol/mg), $K'_m$ is the medium glutamate Michaelis constant (mM),

![Fig. 1. Effect of glutamate concentration on aspartate efflux from aspartate-loaded rat liver mitochondria. Mitochondria were incubated at 10°C in KCl medium, pH 7.2. The data points shown are averages calculated from changes of aspartate in both the mitochondrial matrix and the medium. The solid lines represent the computer fitted curves for each data set.](http://www.jbc.org/)

![Fig. 2. Double reciprocal plots of aspartate efflux kinetics from mitochondria with variation of medium glutamate concentration, calculated according to Equation 5. Glutamate concentrations were: 1.5 mM (A), 2.5 mM (B), 5 mM (C), 10 mM (D), and 15 mM (E).](http://www.jbc.org/)
of increasing the calcium content in the mitochondrial matrix on the kinetics of glutamate-induced aspartate efflux is also shown in Fig. 3. For clarity of presentation, data for only two different calcium loadings are shown. It is readily seen that aspartate efflux was inhibited by preloading the mitochondria with Ca\(^{2+}\).

Using the graphical method of analysis to provide double reciprocal plots, a series of straight lines are produced for the different calcium loadings that intersect at a point close to the abscissa (Fig. 4). Since the intercept of the curves on the ordinate corresponds to the reciprocal of the apparent \(V_{\text{max}}\) (\(V_{\text{max}}\)) it is readily seen that \(V_{\text{max}}\) for aspartate efflux decreases with increased intramitochondrial calcium content (lines A through E in Fig. 4).

In Table III the apparent kinetic constants of the glutamate-aspartate translocator at different levels of mitochondrial calcium loading are compared with simultaneous measurements of the mitochondrial membrane potential (\(\Delta\phi\)) and \(\Delta pH\). Increased calcium loading of the mitochondria was associated with a decrease of \(\Delta\phi\) and an increase of \(\Delta pH\), as previously observed (24, 25). Thus, although \(\Delta\phi\) decreased from 154 mV to 80 mV, \(\Delta pH\) only decreased by 13 mV. Although the \(V_{\text{max}}\) varied over a 5-fold range, the \(K_{m}\) remained almost constant.

### Table I

| Aspartate-loaded rat liver mitochondria (5 mg/ml) were incubated at 10°C in medium containing 100 mM KCl, 20 mM Mops, 8% (w/v) dextran \(M_r = 40,000\), 5 mM ascorbate, and 0.2 mM TMPD, pH 7.2. Aspartate efflux was initiated by addition of glutamate. Kinetic parameters were either calculated from the double reciprocal plots shown in Fig. 2 (Graphical method) or by a computer curve fitting procedure (21). |
|---|---|---|---|---|---|---|---|
| Medium glutamate | Graphical method | Computer method |
| mm | nmol/mg/min | nmol/mg/min | min | nmol/mg/min | nmol/mg/min | min |
| 1.5 | 4.7 ± 0.4 | 5.8 ± 1.4 | 1.24 | 4.1 ± 0.3 | 5.5 ± 0.9 | 1.33 |
| 2.5 | 6.6 ± 0.8 | 7.5 ± 1.8 | 1.10 | 5.8 ± 0.5 | 7.2 ± 0.9 | 0.97 |
| 5.0 | 9.7 ± 1.4 | 4.9 ± 1.0 | 0.50 | 9.4 ± 0.7 | 4.4 ± 0.7 | 0.47 |
| 10.0 | 13.9 ± 1.0 | 5.0 ± 0.8 | 0.36 | 12.5 ± 0.8 | 3.5 ± 0.7 | 0.28 |
| 15.0 | 17.0 ± 1.7 | 5.6 ± 1.1 | 0.33 | 16.4 ± 0.8 | 4.8 ± 0.6 | 0.29 |

### Table II

<p>| Concentration independent kinetic parameters of the glutamate-aspartate translocator |
|---|---|---|---|---|
| Values shown in Table I for the glutamate-dependent kinetic parameters were replotted according to Equations 6 and 7, to give the concentration independent values for the (K_{m}) of external glutamate ((K_{m}^{\text{E}})), the (K_{m}) for internal aspartate ((K_{m}^{\text{I}})) and the (V_{\text{max}}) of the glutamate-aspartate translocator at 10°C. (K) is a mechanism-dependent constant indicative of a ternary carrier complex |</p>
<table>
<thead>
<tr>
<th>Method</th>
<th>(K_{m}^{\text{E}})</th>
<th>(K_{m}^{\text{I}})</th>
<th>(V_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graphical</td>
<td>4.3 ± 0.2</td>
<td>5.3 ± 0.3</td>
<td>21.2 ± 3.5</td>
</tr>
<tr>
<td>Computer</td>
<td>3.9 ± 0.4</td>
<td>6.3 ± 0.7</td>
<td>21.2 ± 3.5</td>
</tr>
<tr>
<td>Average</td>
<td>3.9 ± 0.4</td>
<td>5.8 ± 0.1</td>
<td>21.2 ± 2.0</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Calcium loading (mmol/mg)</th>
<th>(V_{\text{max}})</th>
<th>(K_{m})</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>220 ± 20</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>15</td>
<td>150 ± 20</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>25</td>
<td>100 ± 20</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>35</td>
<td>75 ± 20</td>
<td>6.0 ± 0.8</td>
</tr>
</tbody>
</table>

### Fig. 3

**Effect of mitochondrial calcium loading on aspartate efflux from aspartate-loaded rat liver mitochondria.** Mitochondria were incubated at 10°C in choline chloride medium, pH 7.4, followed by addition of 10 mM glutamate. C, control conditions.

### Fig. 4

**Double reciprocal plots of aspartate efflux kinetics from mitochondria with variation of the mitochondrial calcium content, calculated according to Equation 5.** Calcium contents were: 10 nmol/mg (A), 36 nmol/mg (B), 68 nmol/mg (C), 91 nmol/mg (D), and 111 nmol/mg (E).
The effects of increased extra mitochondrial Ca" on the kinetics of the glutamate aspartate translocator was investigated by adding the same amounts of Ca" to aspartate-loaded mitochondria in the presence of 5 nM ruthenium red, which inhibits energy-linked Ca" uptake (26). Under these conditions, the rate of glutamate-induced aspartate efflux was the same as in the absence of Ca" and ruthenium red (data not shown), suggesting that the inhibitory effect of calcium on aspartate efflux was exerted by the presence of Ca" in the mitochondrial matrix rather than in the medium. This conclusion is supported by the data in Fig. 5 which show that the State 3 rate of respiration in mitochondria incubated with glutamate and malate became inhibited when the mitochondrial Ca" content exceeded 30 nmol/mg of protein.

In order to define the effect of calcium on the kinetics of the glutamate-aspartate translocator in more detail, aspartate-loaded mitochondria were allowed to take up calcium in the presence of acetate. Accumulation of calcium in the presence of a permeant anion allows the charge imbalance to be compensated, and prevents the fall of the mitochondrial membrane potential and alkalinization of the matrix (27, 28, Table IV). In accordance with the results of Table III, Table IV shows that when mitochondria were loaded with calcium in the absence of acetate, the V""max of glutamate-induced aspartate efflux was diminished while the K', remained unchanged. As previously observed, calcium loading was associated with a fall of ΔΨ and increase of ΔpH. With calcium loaded mitochondria in the presence of acetate, the effects of calcium on ΔΨ, ΔpH and the V""max of aspartate efflux were all diminished relative to the controls in the absence of acetate. These data, therefore indicate that matrix Ca" is not interacting directly with the glutamate-aspartate translocator, but is exerting an effect indirectly by a decrease of ΔΨ or increase of the matrix pH.

**Table III**

Effect of intramitochondrial calcium on glutamate aspartate translocator kinetics and mitochondrial membrane potential

Aspartate-loaded mitochondria containing different amounts of calcium were incubated at 10°C, pH 7.4, in choline chloride medium, as described under "Materials and Methods." Aspartate efflux was initiated by addition of 10 mM glutamate. V""max and K"" values were calculated from the double reciprocal plots shown in Fig. 4. ΔΨ is the membrane potential and ΔpH is the proton electrochemical gradient.

<table>
<thead>
<tr>
<th>Calcium content</th>
<th>V&quot;&quot;max</th>
<th>K&quot;&quot;</th>
<th>ΔΨ</th>
<th>ΔpH</th>
<th>ΔpH'</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/mg</td>
<td>mmol/mg</td>
<td>nmol/mg</td>
<td>mV</td>
<td>mV</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>18.5</td>
<td>5.3</td>
<td>153.6</td>
<td>0.79</td>
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<td>36</td>
<td>12.1</td>
<td>5.0</td>
<td>140.2</td>
<td>0.88</td>
<td>193</td>
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<tr>
<td>68</td>
<td>10.1</td>
<td>5.0</td>
<td>126.7</td>
<td>1.19</td>
<td>194</td>
</tr>
<tr>
<td>91</td>
<td>5.8</td>
<td>5.4</td>
<td>104.0</td>
<td>1.47</td>
<td>187</td>
</tr>
<tr>
<td>111</td>
<td>3.5</td>
<td>5.5</td>
<td>80.1</td>
<td>1.87</td>
<td>185</td>
</tr>
</tbody>
</table>

**Fig. 5** Effect of mitochondrial calcium content on the State 3 rate of respiration in rat liver mitochondria incubated with 10 mM glutamate and 1 mM malate.

**Table IV**

Effect of mitochondrial calcium loading in the presence of acetate on glutamate-aspartate translocator kinetics

Aspartate-loaded mitochondria were incubated at 10°C, pH 7.4, in choline chloride medium. Calcium uptake was initiated by addition of 200 μM Ca" in the absence and presence of 10 mM acetate. Efflux of aspartate was followed after addition of 10 mM glutamate. V""max and K"" values were calculated from double reciprocal plots as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Conditions</th>
<th>V&quot;&quot;max</th>
<th>K&quot;&quot;</th>
<th>ΔΨ</th>
<th>ΔpH</th>
<th>ΔpH'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.0</td>
<td>14.4</td>
<td>4.5</td>
<td>153.3</td>
<td>0.84</td>
</tr>
<tr>
<td>+ Ca&quot;</td>
<td>39.8</td>
<td>8.4</td>
<td>4.0</td>
<td>137.2</td>
<td>1.20</td>
</tr>
<tr>
<td>Acetate</td>
<td>7.8</td>
<td>12.5</td>
<td>4.1</td>
<td>134.4</td>
<td>0.60</td>
</tr>
<tr>
<td>Acetate + Ca&quot;</td>
<td>39.7</td>
<td>13.2</td>
<td>5.6</td>
<td>150.4</td>
<td>0.88</td>
</tr>
</tbody>
</table>

**Table V**

Effect of K" plus valinomycin on glutamate-aspartate translocator kinetics and mitochondrial membrane potential

Aspartate-loaded mitochondria were incubated at 10°C, pH 7.4, in choline chloride medium containing valinomycin and K" as indicated. Aspartate efflux was initiated by addition of 10 mM glutamate. V""max and K"" values were calculated from double reciprocal plots as described under "Materials and Methods."

**Table VI**

Effect of K" plus valinomycin on glutamate-aspartate translocator kinetics

Aspartate-loaded mitochondria were incubated at 10°C, pH 7.4, in choline chloride medium containing 0.15 μg/ml of valinomycin. The proton electrochemical gradient was collapsed by addition of 0.15 μg/ml of nigericin. Aspartate efflux was initiated by addition of 10 mM glutamate. V""max and K"" values were calculated from double reciprocal plots as described under "Materials and Methods."

**Effects of Membrane Potential and pH on Aspartate Efflux**—It is well established that the mitochondrial membrane potential can be collapsed by addition of K" in the presence of valinomycin (14). Valinomycin is an uncharged molecule, and forms a positively-charged species when complexed with K" (29). Transport of the lipid-soluble valinomycin-K" complex through the lipid barrier of the inner mitochondrial membrane is thus associated with the electrophoretic movement of K", which tends to collapse the membrane potential (negative inside) established by the outwardly-directed proton pump of the electron transport chain (30). Table V shows the results of increasing medium K" concentrations in the presence of valinomycin on the aspartate efflux kinetic constants. As observed with Ca" (27), which is also transported into mitochondria by an energy-dependent electrophoretic mechanism (31, 32), a decrease of the ΔΨ was associated with an increased ΔpH (14). The V""max for aspartate decreased with diminished steady state levels of ΔΨ while the K" remained approxi-
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Unlike valinomycin, the ionophore nigericin possesses a single charged carboxylic acid group, and promotes an electrical neutral exchange of $K^+$ for $H^+$ (33). When valinomycin and nigericin are both added to mitochondria, the proton permeability of the membrane is increased and a proton electrochemical gradient cannot be maintained. Table VI shows that when both ionophores were added to aspartate-loaded mitochondria incubated in choline chloride buffer, both $\Delta V$ and $\Delta p$ were almost completely collapsed, and the $V_{\text{max}}$ of aspartate efflux was greatly decreased. The $K^*_m$ of aspartate efflux, however, was not affected.

The above data suggest that it is the $\Delta V$ component of the proton electrochemical gradient rather than $\Delta p$ or the matrix $pH$ that is affecting the rate of aspartate transport. When nigericin is added to mitochondria in the presence of high $K^+$ and absence of valinomycin, the $\Delta p$ is collapsed while $\Delta V$ remains little affected (33). Incubation of mitochondria at different external pH values in the absence and presence of nigericin thus allows aspartate transport to be investigated as a function of matrix or medium pH (3). Table VII shows the results of such an experiment with aspartate-loaded mitochondria with media pH values of 6.5 and 7.6. The matrix pH varied from 6.3 to 9.0, but both $V_{\text{max}}$ and $K^*_m$ for aspartate transport remained approximately constant. Although the $V_{\text{max}}$, varied between 12.2 and 14.4 nmol/mg/min, there was no systematic change with either the external or internal pH. Thus, it may be concluded that aspartate transport per se is relatively independent of pH in the experimental pH range.

Results presented above have shown that aspartate efflux was inhibited when the mitochondrial membrane potential was decreased by electrophotopic transport of either Ca$^{2+}$, alone, or $K^+$ in the presence of valinomycin. Fig. 6 shows that there was a linear relationship between $V_{\text{max}}$ of aspartate transport and $\Delta V$ for the pooled results of the two series of experiments, at least over the range of $\Delta V$ from 70 mV to 160 mV. At lower values of $\Delta V$, the relationship was no longer linear.

**TABLE VII**

*Effect of pH on glutamate-aspartate translocator kinetics*

Aspartate-loaded mitochondria were incubated at 10°C in medium containing 100 mM KCl and no valinomycin. Aspartate efflux was initiated by addition of 10 mM glutamate. $V_{\text{max}}$ and $K^*_m$ values were calculated from double reciprocal plots as described under "Materials and Methods." pHo and pHr refer to the medium and mitochondrial matrix pH, respectively. Nigercin was added at a concentration of 0.10 $\mu$g/ml.

<table>
<thead>
<tr>
<th>Ionophore added</th>
<th>$V_{\text{max}}$</th>
<th>$K^*_m$</th>
<th>pHo</th>
<th>pHr</th>
<th>$\Delta p$</th>
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<tbody>
<tr>
<td>None</td>
<td>12.2</td>
<td>3.9</td>
<td>6.5</td>
<td>8.0</td>
<td>1.5</td>
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<tr>
<td>Nigericin</td>
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<td>6.5</td>
<td>6.8</td>
<td>0.2</td>
</tr>
<tr>
<td>None</td>
<td>13.0</td>
<td>3.5</td>
<td>7.6</td>
<td>9.0</td>
<td>1.4</td>
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<tr>
<td>Nigericin</td>
<td>14.4</td>
<td>3.6</td>
<td>7.6</td>
<td>8.0</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**FIG. 6.** Relationship between the apparent $V_{\text{max}}$ of the glutamate-aspartate translocator and the mitochondrial membrane potential (\(\Delta V\)). The open circles (○) are taken from data in Table V, where membrane potential was decreased by K$^+$ plus valinomycin. The open triangles (△) are controls taken from Tables IV and VI, while the solid triangle (▲) represents the de-energized state in Table VI.

**DISCUSSION**

This study arose from an attempt to understand the inhibition of the ADP-stimulated rate of respiration in calcium-loaded mitochondria with glutamate and malate as substrate (see Fig. 5). In order to eliminate possible effects of calcium on the respiratory chain (34, 35), on enzymes of the citric acid cycle, or an aspartate aminotransferase, the present study utilized aspartate-loaded mitochondria in which transaminase activity was fully inhibited by aminoxyacetic acid. Electrogeneic proton efflux was established by permitting electron transport through the third phosphorylation site. Under these conditions, aspartate efflux from the mitochondria is dependent upon addition of extramitochondrial glutamate (3). This study also necessitated the development of methods for resolution of the kinetic parameters from the measured aspartate efflux curves.

The data show that the glutamate-aspartate translocator was inhibited by an increase of calcium in the mitochondrial matrix, but was not affected by the presence of calcium in the incubation medium provided that calcium uptake was prevented. More specifically, the apparent $V_{\text{max}}$ of the translocator was inhibited without significantly changing the apparent $K^*_m$ for matrix aspartate. However, since the inhibitory effect of matrix calcium could be mimicked by increasing $K^+$ permeability by the presence of valinomycin, which like energy driven Ca$^{2+}$ uptake caused a decrease of the mitochondrial membrane potential, it may be concluded that the effect of Ca$^{2+}$ is a secondary phenomenon and does not relate to the presence of calcium per se in the mitochondrial matrix. Under the conditions of the present experiments with limiting K$^+$ or Ca$^{2+}$, the decrease of the membrane potential was accompanied by an increase of $\Delta p$, with the result that the overall proton electrochemical gradient decreased minimally. Theoretically, therefore, inhibition of aspartate transport could be caused by the fall of $\Delta V$ or the increase of the matrix pH. The latter possibility may be considered unlikely because previous data (3) using rat liver mitochondria with the mitochondrial aspartate aminotransferase not inhibited, showed that the aspartate efflux rate increased with an increase of matrix pH over the region from pH 7 to pH 9. Unexpectedly, the present studies showed that the glutamate-aspartate translocator was essentially independent of pH changes in the medium or mitochondrial matrix over this range and was also not affected by a pH gradient of up to 1.5 units. This experimental finding is in agreement with recent data by Dusznyski et al. (18), and suggests that the pH sensitivity of aspartate formation previously observed (3) may actually have reflected the pH sensitivity of aspartate aminotransferase.

An alternative possibility is that aspartate competes with $H^+$ in the matrix for the proton binding site on the carrier. This possibility can be envisioned since the matrix aspartate content is high in the present experiments but is low when aspartate is generated by the matrix aspartate aminotransferase. The model for glutamate-aspartate exchange proposed...
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from previous work (3) was that external glutamate would bind to the protonated form of the carrier on the external surface of the inner mitochondrial membrane, followed by inward transport of neutral glutamate to the matrix. It was further postulated that deprotonation of the carrier would be favored by the alkaline pH of the matrix, thus facilitating the dissociation of the glutamate and binding of aspartate to form a negatively-charged carrier-aspartate complex. Transport of aspartate anion from the matrix to the outer surface of the mitochondrial membrane would then be favored by the membrane potential. However, data presented in this paper suggest that the above model needs modification. First, the dependence of aspartate efflux on ΔΨ rather than on the pH or ΔpH suggests that the rate-limiting step is outward transport of the anionic form of aspartate across the membrane, rather than the rate of deprotonation of the glutamate-carrier complex on the matrix side of the membrane as previously supposed (3). Second, the variation of the $K_m/V_{max}$ ratio with glutamate concentration indicates that a ternary complex is formed between the carrier, glutamate, and aspartate, and that the glutamate-aspartate translocator behaves according to a sequential rather than a Ping-Pong mechanism, as implied from the previous model. These findings are incorporated into a simple minimal model for the glutamate-aspartate translocator shown in Fig. 7. The requirement for a ternary complex suggests that matrix aspartate is bound to the carrier before the release of glutamate. The available data do not provide information whether the form of the carrier after release of aspartate on the cytosol side of the membrane is the same as that which binds glutamate, as shown in the model, but it is evident that if this step occurs it would not be kinetically rate limiting. For energized mitochondria where there is an electrical potential across the mitochondrial inner membrane (negative inside), directionality of transport is achieved by the kinetically rate-limiting step of transport of the charged carrier-aspartate complex from the matrix to the cytosol side of the membrane.

**Fig. 7. Model of the glutamate-aspartate translocator.** The symbol C represents the mobile carrier in the membrane, while HGlua represents the undissociated form of glutamate, which is assumed to bind directly to the carrier.

Derivation of the concentration-independent kinetic constants for glutamate-aspartate transport permits the formulation of a rate equation to describe aspartate efflux from the mitochondria. The rate equation given below assumes irreversible transport and that extramitochondrial aspartate is a noncompetitive inhibitor (3). Thus, the velocity ($v$) is a function of the three variables, $A_o$ (external aspartate concentration), $A_m$ (matrix aspartate content), and $G_o$ (external glutamate concentration).

$$v = \frac{1}{V_{max}} \frac{1}{V_{max}} (1 + A_o/K_i) + \frac{K_m}{V_{max}} - G_o \frac{1 + A_o}{K_i}$$

where $V_{max}$ is the maximum velocity at 10°C, $K_i$ is the noncompetitive inhibition constant for external aspartate which is taken as $4 \pm 1$ mM (3), $K_m$ is the Michaelis constant for external glutamate, $K_m^*$ is the Michaelis constant for matrix aspartate, and $K_i$ is a mechanism-dependent constant (see Table II). The velocity of aspartate efflux at 10°C calculated from Equation 8 may then be converted to other temperatures by using the previously determined value of 15.1 kcal/mol for the energy of activation of the glutamate-aspartate translocator (3). Illustrations of the use of Equation 8 to calculate aspartate efflux from mitochondria using data from isolated rat hepatocytes at 37°C are given in Table VIII. Flux units of nmol/mg of mitochondrial protein/min calculated from Equation 8 were converted to units of μmol/g dry weight of liver/h by assuming a mitochondrial content of 240 mg/g dry weight.

As discussed in detail elsewhere (9), when liver cells are incubated with 10 mM DL-cycloserine, ammonia, and lactate, the cytosolic aspartate aminotransferase is completely inhibited so that the rate of the glutamate-aspartate exchange across the mitochondrial membrane can be accurately calculated from the sum of the rates of urea production and aspartate accumulation after 30 min of incubation. Mitochondrial aspartate efflux was also estimated from Equation 8 using constants shown in Table II.

**TABLE VIII**

Comparison of measured and calculated rates of mitochondrial aspartate efflux using isolated rat hepatocytes

Liver cells (4 to 5 g dry weight/ml) isolated from 18 to 24 h fasted rats (36) were incubated at 37°C in Krebs bicarbonate medium, pH 7.4, containing 4% bovine serum albumin and the following substrates. Medium A contained 10 mM L(+)-lactate, 1 mM oleate, 5 mM ammonia, and 10 mM DL-cycloserine. Medium B contained the same substrates plus 3 mM ornithine. In other experiments, oleate was present at 1 mM and lactate or pyruvate at 5 mM. Mitochondria were rapidly separated from the cells by the cell disruption technique of Tischler et al. (37, 38) for assay of mitochondrial and cytosolic glutamate and aspartate. Aspartate efflux for experiments with Medium A and B was estimated from the sum of the rates of urea production and aspartate accumulation after 30 min of incubation. Mitochondrial aspartate efflux was also estimated from Equation 8 using constants shown in Table II.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>[Asp], m M</th>
<th>[Glu], mM</th>
<th>Δ Urea + Δ aspartate</th>
<th>Aspartate efflux from rate equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>m M</td>
<td>m M</td>
<td>μmol/ml</td>
<td>μmol/g dry wt/h</td>
<td></td>
</tr>
<tr>
<td>Medium A, Experiment 1</td>
<td>10.7</td>
<td>27.5</td>
<td>14.1</td>
<td>513</td>
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<tr>
<td>Medium A, Experiment 2</td>
<td>10.6</td>
<td>17.3</td>
<td>19.8</td>
<td>824</td>
</tr>
<tr>
<td>Medium B, Experiment 1</td>
<td>5.0</td>
<td>21.3</td>
<td>3.2</td>
<td>620</td>
</tr>
<tr>
<td>Medium B, Experiment 2</td>
<td>2.7</td>
<td>15.2</td>
<td>4.0</td>
<td>986</td>
</tr>
</tbody>
</table>

Lactate + oleate 0.0 2.6 1.4 169 ± 17
Pyruvate + oleate 0.4 1.8 0.6 68 ± 6

The 32 values with mean ± standard error of the mean were calculated by a computer program.
that the agreement between the measured and calculated rates of aspartate efflux from the mitochondria is remarkably good, considering the large number of parameters involved in the calculations. Furthermore, the range of experimental values allowed the rate equation to be tested at values of cytosolic aspartate below and above the \( K_a \) for aspartate inhibition, and values of mitochondrial aspartate also below and above the \( K_m \) for aspartate. The last two lines of Table VIII show rates of mitochondrial aspartate transport calculated for conditions with lactate and pyruvate, respectively, as gluconeogenic substrates where both glutamate and aspartate concentrations were below their respective \( K_m \) values. It is generally accepted that glutamate binds to the carrier as the undissociated acid.

It is apparent from the above illustration of the application of the glutamate-aspartate translocator rate equation to data obtained with intact hepatocytes that the kinetic mechanism for aspartate transport is now reasonably well described. Differences between measured and calculated fluxes can probably be ascribed to experimental errors in the determination of the glutamate and aspartate concentrations or their kinetic constants. It would appear unnecessary, therefore, to invoke the possibilities of direct interaction between the glutamate-aspartate carrier and aspartate aminotransferase (3) or a large diffusion gradient of aspartate within the mitochondrial matrix (18). The discrepancy between our value of 4.9 mM for the matrix aspartate \( K_m \) with isolated mitochondria, and the value of 38 \( \mu \)M for the external aspartate \( K_m \) with submitochondrial particles observed by Duszynski et al. (18), possibly can be ascribed to alterations induced in the mitochondrial membrane during preparation of the particles.

Note Added in Proof—Attention is drawn to the paper by F. Palmieri et al. (Biochim. Biophys. Acta. (1979) in press) which shows that L-cysteinesulfinic acid is also transported on the glutamate-aspartate carrier. Data in this paper support the suggestion that glutamate binds to the carrier as the undissociated acid.

REFERENCES

Kinetics and regulation of the glutamate-aspartate translocator in rat liver mitochondria.
E Murphy, K E Coll, R O Viale, M E Tischler and J R Williamson