Ammonia Formation in Isolated Rat Liver Mitochondria*

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Studies of isolated rat liver mitochondria were undertaken in order to evaluate the importance of glutamate transport, oxidation reduction state, and product inhibition on the rates of formation of ammonia from glutamate. Uptake and efflux of glutamate across the mitochondrial membrane were measured isotopically in the presence of rotenone. Efflux was stimulated by H⁺ in the mitochondrial matrix and was found to be first order with respect to matrix glutamate except when the matrix pH was unphysiologically low. The data suggest that the Kₘ of matrix glutamate for efflux is decreased by H⁺. Matrix H⁺ also appeared to stimulate glutamate uptake, and the effect was to increase both the Kₘ of medium glutamates and Vₘₐₓ.

Mitochondria were incubated at 15 and 28 °C with glutamate and malonate. Under these conditions, glutamate was metabolized only by the deamination pathway. Flux was evaluated by assay of ammonia formation. Oxidation reduction state was varied with ADP and uncoupling agents. Matrix α-ketoglutarate was varied either by the omission of malonate from the incubation media or by adding α-ketoglutarate to the external media. Influx and efflux of glutamate could be calculated from previously determined transport parameters. The difference between calculated influx and efflux was found to be equal to ammonia formation under all conditions. It was, therefore, possible to evaluate the relative contributions of oxidation reduction state, transport, and product inhibition as effectors of ammonia formation. The contribution of transport was relatively small while oxidation reduction state exerted a large influence. α-Ketoglutarate was found to be a potent competitive inhibitor of ammonia production and glutamate dehydrogenase. Inhibition of glutamate dehydrogenase by α-ketoglutarate was judged to be a potentially important modulator of metabolic fluxes.

The mechanism of control of glutamate dehydrogenase has been the subject of many experimental studies in recent years (1, 2). It is of interest because it catalyzes an important, possibly rate-controlling step in several metabolic pathways, including gluconeogenesis from amino acids, and ammoniagenesis in the kidney (6). The enzyme is also of interest to enzymologists because it is a multisubunit complex subject to a wide variety of allosteric controls (7, 8). Many attempts have been made to apply what is known about the control properties of the isolated enzyme to flux in the metabolic pathways. For example, since leucine is an allosteric stimulator of glutamate dehydrogenase, it was suggested (9, 10) that leucine levels in the cell may exert a significant influence on glutamate dehydrogenase flux. However, this effect of leucine has been shown not to be physiologically important (11).

It is well established that glutamate dehydrogenase is located exclusively in the mitochondria (12). Therefore, studies of its properties are in theory somewhat simplified since flux may be measured in suspensions of isolated mitochondria where control of physiological effectors is feasible. Additionally, measurements of intraand extramitochondrial metabolites and cofactors is a technically easier task than in the intact organ or in isolated hepatocytes. Yet studies of the activity of glutamate dehydrogenase in isolated mitochondria have perhaps raised more questions than they have answered. Despite the unquestioned activity and flux through glutamate dehydrogenase in the intact cell, ammonia formation from glutamate in isolated mitochondria is usually negligible, although it can be stimulated by uncoupling agents (13, 14). Under coupled conditions in isolated mitochondria, almost all glutamate is metabolized by the transaminase pathway.

An important assumption made in many studies of mitochondrial metabolism is that the mitochondria behave the same in isolation as they do in the cell and that isolated mitochondrial matrix enzymes behave similarly in isolation and in situ. However, in the case of glutamate dehydrogenase, the assumption is questionable. It has been shown that glutamate dehydrogenase is bound to the mitochondrial inner membrane and inhibited by the mitochondrial phospholipid, cardiolipin (15, 16). Additionally, the isolated enzyme uses both NAD and NADP as cofactors, but the enzyme in situ appears to be specific for NADP (17, 18). This conclusion is based on the observation that in coupled mitochondria NADP is very low and glutamate dehydrogenase activity is low whereas NADP increases dramatically when mitochondria are uncoupled and enzyme activity is high. The NADP(H) redox state is much more profoundly altered by uncoupling than the NAD(H) redox state due to the activity of the energy-linked transhydrogenase reaction. On the other hand, equilibrium studies with isolated mitochondria suggest that the mass action ratio is more sensitive to free NAD/NADH ratios than measured NADP/NADPH ratios (19), suggesting that the enzyme prefers NAD to NADP as cofactor.

Suggestions have also been made that the dehydrogenase is controlled entirely by substrate availability in liver mitochondria, because transport of glutamate into liver mitochondria is an order of magnitude slower than maximal rates of glut-
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MATERIALS AND METHODS

Mitochondrial isolation and incubation: Rat liver mitochondria were prepared from male Sprague-Dawley rats weighing 250-350 g. The mitochondria were isolated by standard techniques of different centrifugation (25) in a buffer (pH 7.2) containing 225 mM mannitol, 5 mM KH$_2$PO$_4$, and 5 mM EDTA. The mitochondria were suspended in the assaying media at a concentration of 5 mg of protein/ml and used within 5-6 hours. Incubations were carried out at 0°C in a medium containing 150 mM KC1, 5 mM KH$_2$PO$_4$, 10 mM HEPES, and 15 mM succinate. Variations of the medium were made as noted in the figures legends. The respiratory control ratio of the mitochondria was determined polarographically with a Clark electrode. The respiratory control ratio of each mitochondrial preparation was tested by measuring oxygen consumption at 0.5 mM ADP in a medium composed of 150 mM KCl, 5 mM KH$_2$PO$_4$, 20 mM HEPES, and 50 mM glucose. Mitochondria usually had respiratory control ratio of 8-9. No preparation with a ratio less than 5 was used.

In order to measure transport and intermitochondrial metabolic levels, mitochondria were usually isolated from rat livers using a modification of the silica gel centrifugation method (26). The mitochondrial pellet was resuspended in a medium composed of 225 mM mannitol and 10 mM HEPES, pH 7.2, and the final concentration of the mitochondrial suspension was kept at 5 mg of protein/ml.

Validation of the methodology of measuring glutamate transport: Contradictory data (25-29) appear in the literature regarding the kinetics parameters of glutamate dehydrogenase in rat liver. The mitochondria were resuspended in a medium containing 50 mM KC1, 5 mM KH$_2$PO$_4$, 10 mM HEPES, and 50 mM glucose, pH 7.2, and the final concentration of the mitochondrial suspension was kept at 5 mg of protein/ml. Mitochondria usually had respiratory control ratio of 8-9. No preparation with a ratio less than 5 was used.

The hyperpolarization F$_{0}$ was measured from the electrical activity of the mitochondrial adenine nucleotide translocase. The translocase activity was assayed using the assay described by Hokin and Hubley (30). The translocase activity was measured polarographically with a Clark electrode. The respiratory control ratio of each mitochondrial preparation was tested by measuring oxygen consumption at 0.5 mM ADP in a medium composed of 150 mM KCl, 5 mM KH$_2$PO$_4$, 20 mM HEPES, and 50 mM glucose. Mitochondria usually had respiratory control ratio of 8-9. No preparation with a ratio less than 5 was used.

Figure 3. Kinetics of glutamate influx into rat liver mitochondria at different matrix pH values. Kinetics of glutamate influx were determined in a medium of pH 7.2 containing 150 mM KC1, 5 mM KH$_2$PO$_4$, 15 mM succinate, 5 mM EGTA, and 5 mM MgATP. Temperature was maintained at 25°C. Mitochondria were isolated from the liver of Sprague-Dawley rats. The initial reaction rate was determined from the increase in glutamate uptake after addition of glutamate to the medium. The results were determined from the 1,2,4,6-tetracyanobenzoic acid (TcA) procedure.
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**RESULTS**

Glutamate Transport at 15°C—Data summarizing the results of three experiments measuring glutamate uptake and glutamate efflux at 15°C are shown in Table I. The experimental methods and calculations used to obtain these rate constants (k) and fluxes (V) are described in detail in the Miniprint. The Miniprint also contains the theoretical basis and experimental demonstration of the fact that the rate constants obtained from glutamate uptake kinetics are the same as the first order rate constants of glutamate efflux from the mitochondria. The data of Table I show that the rate constants obtained from uptake experiments and from efflux experiments are statistically the same. This validates the method of calculating fluxes in both directions across the mitochondrial membrane. Acidification of the matrix causes a profound increase in the efflux rate constants, as might be expected from a carrier dependent on proton co-transport. However, the stimulation of influx by matrix H+ is surprising.

The kinetic parameters of glutamate transport were also measured as a function of external glutamate and matrix pH. The results are shown in Fig. 4. Values are shown are means ± SE for three to four separate determinations. Values obtained at glutamate levels below 6 nmol/mg mitochondrial protein.

**TABLE I**

Kinetic parameters of glutamate-OH exchange in rat liver mitochondria

<table>
<thead>
<tr>
<th>Condition</th>
<th>Matrix pH</th>
<th>k' uptake</th>
<th>k' efflux</th>
<th>V = k' (Gr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.20</td>
<td>0.080 ± 0.006</td>
<td>0.076 ± 0.006</td>
<td>0.470</td>
</tr>
<tr>
<td>+5 mM</td>
<td>7.80</td>
<td>0.123 ± 0.085</td>
<td>0.153 ± 0.061</td>
<td>0.570</td>
</tr>
<tr>
<td>+ FCCP</td>
<td>7.55</td>
<td>1.11 ± 0.21</td>
<td>0.80 ± 0.02*</td>
<td>1.76 (1.28)*</td>
</tr>
</tbody>
</table>

* Values obtained at glutamate levels below 6 nmol/mg mitochondrial protein.

**FIG. 4.** The effect of matrix pH on the kinetics of mitochondrial glutamate transport. The experimental conditions were the same as those described for Fig. 2. The temperature was 15°C and the glutamate concentrations were varied as shown. Kinetic parameters obtained for the control condition in the absence of phosphate (○) were as follows: V_{max} was 1.75 nmol/min-mg while K_{m} was 4.75 mM. In the presence of phosphate (△), V_{max} was 3.48 nmol/min-mg and K_{m} was 8.93 mM. The average efflux rate constants in this experiment were 0.096 min^{-1} in the control condition and 0.11 min^{-1} in the presence of phosphate.
glutamate. The data would imply that return of the free carrier to the external glutamate-binding conformation is the rate-limiting step in transport. If so, exchange transport should be significantly more rapid than net transport.

The rate of exchange transport was measured and compared directly with net uptake in the experiment of Fig. 5. Mitochondria were incubated at 15°C with 2 mM unlabeled glutamate. Samples were taken at the times shown to determine by enzymatic assay the net rate of uptake of unlabeled glutamate. At 21 min, a tracer amount of [14C]glutamate was added to the incubation media and samples were taken at 15–20-s intervals to determine the rate of glutamate/glutamate exchange. The data show that glutamate/glutamate exchange is about 3 times faster than net transport. Control experiments (not shown) indicated that this exchange could be inhibited by N-ethylmaleimide and, therefore, was not due to the activity of the glutamate-aspartate transporter. Rapid exchange rates may explain why most of the uptake curves do not pass through the origin of the graphs (cf. Fig. 2). Initial (0–10 s) rates of transport were significantly faster than subsequently calculated rates. Determination of the specific activity of matrix glutamate at very early time points suggests that these rapid initial rates may be accounted for by exchange with endogenous glutamate.

**Measurements of Glutamate Deamination at 15°C**—The next step in this study was to relate the transport of glutamate to its metabolism. The transport rate of glutamate and the oxidoreduction state of mitochondrial NAD(H) were varied in a controlled manner and the effects on ammonia formation were measured.

In order to simplify the analysis of the data, metabolism of glutamate via the transaminase pathway was prevented by including malonate in the incubation media. Malonate inhibits succinate dehydrogenase so that the carbon from glutamate oxidation cannot generate oxalacetate via the citric acid cycle. This depletes the mitochondria of oxalacetate and thus prevents transamination of glutamate with oxalacetate. Four conditions designed to alter the NADH/NAD ratio of the mitochondrial matrix were studied. These included the controlled state, in which glutamate but not ADP was present (state 4) and NADH is high, the ADP-stimulated state (state 3) in which NADH is lower, the uncoupled state where NADH is lower yet, and the rotenone-inhibited state in which metabolism of glutamate was prevented and NAD(H) was completely reduced. In each case, 5 mM inorganic phosphate and 5 mM Mg2+ were present. The results are shown in Table II. In these two experiments, the glutamate concentration was varied 10-fold to examine the influence of the rate of glutamate transport across the mitochondrial matrix on ammonia formation. In the rotenone-inhibited state, [14C]glutamate was used, but in other experiments, glutamate was not radioactive. Because of the technical problems involved in measuring the rate constant of glutamate uptake in the presence of uncoupler, transport was only measured in the coupled plus phosphate state. Samples were taken as indicated in the legend of Table II and the mitochondria rapidly separated from their media. Matrix glutamate levels were measured enzymatically and the steady state levels attained after 5 min in the absence of rotenone are reported in Table II. In the rotenone-inhibited state, the steady state matrix glutamate (Gt) is obtained in

### Table II

<table>
<thead>
<tr>
<th>Condition</th>
<th>Glutamate</th>
<th>Meas-</th>
<th>Calcu-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>External</td>
<td>Matrix</td>
<td>Ef-</td>
</tr>
<tr>
<td></td>
<td>mmol/mg</td>
<td>mmol/min/mg</td>
<td>flux</td>
</tr>
</tbody>
</table>
|                            |           |                | k
| State 4                    | 4.5       | 7.25           | 1.37            | 0.40  | 1.17            |
|                            | 0.45      | 0.24           | 0.05            | 0.15  | 0.17            |
|                            | 4.5       | 2.50           | 0.45            | 1.51  | 1.47            |
|                            | 0.45      | 0.00           | 0.50            | 0.50  | 0.50            |
|                            | 4.5       | 1.00           | 1.1             | 2.17  | 3.27            |
|                            | 0.45      | 0.15           | 0.165           | 0.50  | 0.66            |
|                            | 4.5       | 7.82           | 1.47            | 0.50  | 1.47            |
|                            | 0.45      | 0.94           | 0.176           | 0.176 | 0.176           |
| State 3                    | 11.1      | 16.5           | 4.45            | 0.50  | 4.90            |
|                            | 1.08      | 1.48           | 0.414           | 0.24  | 0.67            |
|                            | 11.1      | 7.5            | 2.02            | 2.10  | 4.90            |
|                            | 1.08      | 0.496          | 0.139           | 0.59  | 0.67            |
|                            | 11.1      | 2.54           | 2.79            | 2.70  | 5.50            |
|                            | 1.08      | 0.27           | 0.28            | 0.80  | 1.10            |
|                            | 11.1      | 18.5           | 4.90            | 0.50  | 4.90            |
|                            | 1.08      | 2.40           | 0.67            | 0.67  | 0.67            |

**a Steady state.**
the usual way from uptake curves similar to those shown in Fig. 2. The first order rate constant of efflux was obtained by measuring the uptake of [14C]glutamate in the presence of rotenone. When the efflux rate constant is multiplied by the steady state matrix glutamate levels, the rate of efflux of glutamate at steady state is obtained. Likewise, the uptake curve in the presence of rotenone will provide data on the rate of glutamate entry. Separate experiments indicated that matrix pH did not vary significantly, depending on whether succinate plus rotenone was present or glutamate plus malonate. The last column of Table II is the rate of influx of glutamate calculated by adding the rate of efflux to the rate of ammonia formation. This calculated influx should equal the measured influx from the rotenone experiment. It can be seen from the data of Table II that the calculated influx and measured influx agree reasonably well. Ammonia formation varies 3-6-fold according to the NAD(H) redox state at constant rates of glutamate influx. This suggests that transport of substrate is not completely rate limiting for glutamate dehydrogenase since influences due to the control of the enzyme can be observed. Likewise, it is apparent that under most metabolic conditions a significant fraction of the entering glutamate is not metabolized further, but instead is transported out of the matrix. Another interesting aspect of the data is the fact that ammonia formation is only slightly faster in the uncoupled state than in state 3, a result very different from previous studies in which malonate was not included in the media. Although the data of Table II and Fig. 2 show that glutamate transport is faster in the presence of uncoupler, it is not the increased rate of glutamate transport which causes the increase in ammonia formation. If increased transport were solely responsible for increased ammonia formation, matrix glutamate levels would be higher in the presence of uncoupler. The fact that ammonia formation is faster in the face of lowered matrix glutamate levels demonstrates the importance of the oxidation of the pyridine nucleotides caused by the uncoupling agent.

Despite the fact that the activity of glutamate dehydrogenase is obviously an important variable, the supply of glutamate also has an influence. However, the rate of ammonia formation is far less sensitive to changing external glutamate than is the rate of glutamate transport. There is a significant competition between glutamate dehydrogenase and the efflux pathway. Since these two processes have separate activation energies and could be influenced differentially by temperature, it seemed important to carry out further more detailed metabolic studies at a more physiological temperature while still obtaining sufficient temporal resolution to obtain accurate transport kinetic parameters. Therefore, all further experiments were performed at 28 °C.

Glutamate Transport at 28 °C—Preliminary experiments similar to those shown in Table II carried out at 28 °C indicated serious discrepancies between calculated influx rates and measured influx rates. In fact, ammonia formation was faster than measured influx. It became apparent that these discrepancies were due to artifacts introduced by the presence of Mg2+ in the incubation media. Although Mg2+ has no apparent effect on uptake or efflux at 15 °C (data not shown), it does appear to influence uptake at 28 °C.

The influence of Mg2+ and phosphate on glutamate uptake is illustrated in Fig. 6. Mg2+ unexpectedly appears to inhibit glutamate uptake in the presence of phosphate. However, separate experiments showed that glutamate respiration in state 3 with malonate was not effected by Mg2+ even when glutamate levels were lowered well below the Km for respiration (1.1 mM). Swelling studies showed, however, that mitochondria swell continuously in the presence of Mg2+ and phosphate at 28 °C but not at 15 °C. Therefore, we suspected that the problem was an artifact due to changing matrix volumes during the course of the uptake experiments. To check on this possibility, efflux was measured directly from glutamate-loaded mitochondria in the presence and absence of Mg2+ (Fig. 7). Mg2+ does not alter the rate of efflux of glutamate from mitochondria. The experiments of Figs. 6 and 7 were repeated in five different preparations of isolated rat liver mitochondria. A summary of the calculated transport parameters is shown in Table III. In the absence of Mg2+, with or without phosphate, the uptake rate constant is equal to the efflux rate constant. However, when phosphate and Mg2+ are present, the uptake rate constant is significantly slower than the efflux rate constant. It was, therefore, assumed that the uptake rate constant is an artifact and the measured efflux rate constant was used in subsequent calculations of transport. If one assumes linear Michaelis-Menten kinetics for transport in the inward direction, the two data points shown in Table III for 10 and 1 mM glutamate (in the presence of phosphate and Mg2+) can be used to obtain values for Km and Vmax. At 28 °C, the calculated Vmax is 3.11 nmol/min·mg and the Km is 9.3 mM, in fairly good agreement with the data of Bradford and McGivan (20).
Ammonia Formation at 28 °C—Further experiments were performed in order to elucidate the relationships between glutamate transport and glutamate dehydrogenase flux at 28 °C. Experiments performed at 15 °C suggested that glutamate dehydrogenase is significantly influenced by the presence of malonate in the incubation media. It seemed likely that this occurred because malonate prevents the formation of an excess of α-ketoglutarate via the glutamate transaminase pathway. In order to confirm this conclusion, some experi-

![Graph](image)

**Fig. 7.** The effect of Mg2+ on glutamate efflux at 28 °C. Mitochondria were loaded with [14C]glutamate by incubating the mitochondria (30 mg/ml) with [14C]glutamate (50 mM, 4 µCi), 200 mM sucrose, 10 mM succinate, 10 µM rotenone, 10 mM MOPS at pH 7.0 at 0 °C for 1 h. The mitochondria were rapidly separated from the media at 0 °C by centrifugation, washed twice in sucrose-mannitol isolation media, and resuspended. The washed, glutamate-loaded mitochondria (4 mg/ml) were added to phosphate buffers (28 °C) identical with those described in Fig. 6A either with or without 5 mM MgCl2. Samples were taken from the incubation media as shown and mitochondrial ammonia formation was determined. Some mitochondrial extracts were assayed enzymatically for glutamate dehydrogenase isopycnic activity, ammonia, and glutamate. The matrix samples were assayed in the supernatant. Some mitochondrial extracts were assayed enzymatically for glutamate and all were assayed for radioactivity.

![Graph](image)

**Fig. 8.** Mitochondrial ammonia production. A, the influence of oxidation reduction state and malonate. Mitochondria (8 mg/ml) were incubated at 28 °C in a buffer (pH 7.0), containing 130 mM KCl, 20 mM MOPS, 5 mM KH2PO4, 20 mM glucose, 5 mM MgCl2, 7% dextran, [14C]sucrose (0.4 µCi/ml), 18O (4.0 µCi/ml), and either 10 (open symbols) or 1 mM (closed symbols) glutamate. In some incubations (state 3 + malonate), hexokinase (8 units/ml) and malonate (5 mM) were also included in the buffer. In state 4, the only addition to the basic media was 5 mM malonate. In **STATE 3, NO MALONATE**, hexokinase (8 units/ml) was added but no malonate. Samples were taken as shown and mitochondria were separated from the media by centrifugation through silicone oil. The media was assayed for radiactivity, ammonia, and glutamate. The matrix samples were assayed for radioactivity and glutamate. B, the influence of α-ketoglutarate (α-KG) on mitochondrial ammonia formation. Incubation conditions were the same as those described in A, **STATE 3 + MALONATE**, but α-ketoglutarate was also included to the incubation media. Initial media concentrations of α-ketoglutarate are shown. In one incubation (open symbols), 10 mM glutamate was present and in another (closed symbols), 1 mM glutamate was present. Ammonia formation (circles) and media α-ketoglutarate levels (squares) were assayed in the supernatant.

**TABLE III**

<table>
<thead>
<tr>
<th>P. Mg2⁺ Rate constants</th>
<th>Rate constants</th>
<th>Transport rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{i0}$</td>
<td>$k_i$</td>
<td>$k_v$</td>
</tr>
<tr>
<td>$\text{nmol} / \text{mg}$</td>
<td>$\text{nmol} / \text{mg}$</td>
<td>$\text{nmol} / \text{mg}$</td>
</tr>
<tr>
<td>+ +</td>
<td>0.375 ± 0.032</td>
<td>0.334 ± 0.026</td>
</tr>
<tr>
<td>+ -</td>
<td>0.94 ± 0.054</td>
<td>0.90 ± 0.048</td>
</tr>
<tr>
<td>- -</td>
<td>0.58 ± 0.093</td>
<td>0.52 ± 0.062</td>
</tr>
</tbody>
</table>
Effect of transport, oxidoreduction potential, and α-ketoglutarate on glutamate metabolism

Mitochondria were incubated under conditions identical with those described in Fig. 8 except for the concentration of α-ketoglutarate which was initially 1 mM. Malonate was omitted from incubations labeled "No malonate." When present, hexokinase was 8 units/ml and malonate was 5 mM. Temperature was maintained at 28 °C. Samples were taken as shown in Fig. 8. Values shown are means ± SE of five separate experiments. No calculations of influx or efflux are made in the no malonate condition since transport via glutamate-aspartate exchange is possible in this situation.

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<table>
<thead>
<tr>
<th>Condition</th>
<th>Media glutamate</th>
<th>ΔNADH</th>
<th>Matrix glutamate</th>
<th>Efflux</th>
<th>Influx</th>
<th>ΔNADH + Efflux</th>
<th>NADH/NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3</td>
<td>10</td>
<td>5.73 ± 0.37</td>
<td>10.12 ± 0.64</td>
<td>9.34</td>
<td>16.7 ± 0.80</td>
<td>15.07</td>
<td>0.345</td>
</tr>
<tr>
<td>State 4</td>
<td>10</td>
<td>2.12 ± 0.54</td>
<td>14.53 ± 1.26</td>
<td>13.37</td>
<td>16.7</td>
<td>15.49</td>
<td>0.658</td>
</tr>
<tr>
<td>No malonate</td>
<td>10</td>
<td>1.31 ± 0.35</td>
<td>20.90 ± 3.44</td>
<td>14.63</td>
<td>16.7</td>
<td>16.95</td>
<td>0.307</td>
</tr>
<tr>
<td>+α-Ketoglutarate</td>
<td>10</td>
<td>2.32 ± 0.22</td>
<td>15.90 ± 0.9</td>
<td>14.63</td>
<td>16.7</td>
<td>16.95</td>
<td>0.307</td>
</tr>
<tr>
<td>State 3</td>
<td>1</td>
<td>3.04 ± 0.23</td>
<td>1.04 ± 0.27</td>
<td>0.96</td>
<td>3.15 ± 0.25</td>
<td>4.08</td>
<td>0.089</td>
</tr>
<tr>
<td>State 4</td>
<td>1</td>
<td>1.71 ± 0.26</td>
<td>2.71 ± 0.64</td>
<td>2.49</td>
<td>3.15</td>
<td>4.20</td>
<td>0.226</td>
</tr>
<tr>
<td>No malonate</td>
<td>1</td>
<td>0.65 ± 0.20</td>
<td>2.6 ± 0.81</td>
<td>2.52</td>
<td>3.15</td>
<td>3.7</td>
<td>0.386</td>
</tr>
<tr>
<td>+α-Ketoglutarate</td>
<td>1</td>
<td>0.75 ± 0.27</td>
<td>2.74 ± 0.45</td>
<td>2.52</td>
<td>3.15</td>
<td>3.7</td>
<td>0.386</td>
</tr>
</tbody>
</table>

Kinetic parameters of glutamate dehydrogenase in liver mitochondrial sonate

The reaction media was pre-equilibrated at 28 °C for 5 min and then 0.1 mg of mitochondrial protein was added from a mitochondrial sonate prepared as described under "Materials and Methods." The buffer used contained 1 mM NAD, 120 mM KCl, 10 mM MOPS, 2 mM ADP, 1 mM EDTA and was adjusted to either pH 7.6 or 7.1. Initial rates of formation of NADH were monitored on an Eppendorf fluorometer.

<table>
<thead>
<tr>
<th>Glutamate Kₚ</th>
<th>Vₜₐₐₙ</th>
<th>pH 7.6</th>
<th>pH 7.1</th>
<th>pH 7.6 + NADH (44.6 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol/min-mg</td>
<td>mmol/min-mg</td>
<td>9.80 ± 0.10</td>
<td>78.65 ± 2.7</td>
<td>9.77 ± 0.05</td>
</tr>
<tr>
<td>mmol/min-mg</td>
<td>mmol/min-mg</td>
<td>9.71 ± 0.08</td>
<td>40.0 ± 1.8</td>
<td>9.77 ± 0.05</td>
</tr>
</tbody>
</table>

Control of Ammonia Formation in Sonicated Mitochondria—In order to estimate the sensitivity of glutamate dehydrogenase to NADH and α-ketoglutarate, flux through glutamate dehydrogenase in the direction of deamination was measured in sonicated mitochondria. Mitochondria were sonicated in a medium composed of 50 mM KH₂PO₄, 5 mM MgCl₂ at pH 7.5. Sonication was carried out for 2 min at 0 °C (50% duty cycle) with a Branson Sonifier. The sonicate was centrifuged at 14,000 × g for 15 min to sediment unbroken mitochondria. The supernatant solution was used to assay glutamate dehydrogenase and mitochondrial protein. The conditions of assay were similar to those used by McGivan et al. (30) but the assay was performed at 28 °C in a temperature-equilibrated Eppendorf fluorometer. Initial rates of increase in fluorescence due to the appearance of NADH were used as measures of enzymatic activity. The effects of glutamate concentration, pH, NADH, and α-ketoglutarate were measured. The data are shown in Table V and Fig. 9. A decrease in media pH from 7.6 to 7.1 decreased Vₚₚₚₚ by about 50% without an appreciable effect on Kₚ. The Vₚₚₚₚ was also sensitive to micromolar quantities of NADH even though the assay was performed in the presence of 1 mM NAD. The Kₚ was 9.8 mm and Vₚₚₚₚ in the absence of inhibitors was 78.6 nmol/min-mg. This compares well with the value of 55 nmol/min-mg obtained by McGivan et al. (30) under similar conditions. The Dixon plot used to calculate the Kₚ for α-ketoglutarate is shown in Fig. 9. The Kₚ of α-ketoglutarate for liver mitochondrial glutamate dehydrogenase measured here (0.35 mM) is somewhat lower than that measured recently for kidney mitochondrial glutamate dehydrogenase (0.6 mM) (24). The fact that initial rates of glutamate deamination in the absence of added ammonia are inhibited by α-ketoglutarate suggests that α-ketoglutarate is a competitive inhibitor of glutamate binding to the enzyme and that it is not simply exerting its effect on flux by fueling the reverse reaction.

The abbreviations used are: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MOPS, 4-morpholinepropanesulfonic acid; DMO, 5,5'-dimethyloxazolidine-2,4-dione.
The temperature was 28 °C and the pH of the media was 7.6. The glutamate concentration used in the incubations was either 20.8 mM (A), 6.92 mM (B), or 5.53 mM (C). α-KG, α-ketoglutarate.

**Fig. 9.** The influence of α-ketoglutarate on the activity of glutamate dehydrogenase in sonicated mitochondrial extracts. Incubation conditions are the same as those described in Table V. The temperature was 28 °C and the pH of the media was 7.6. The glutamate concentration used in the incubations was either 20.8 mM (A), 6.92 mM (B), or 5.53 mM (C). α-KG, α-ketoglutarate.

**DISCUSSION**

Two important deductions can be made from the data. First, glutamate transport across liver mitochondrial membranes is probably not a significant rate-limiting step for ammonia formation. It is true that at 28 °C the apparent $V_{\text{max}}$ of transport is lower (31 nmol/min-mg) than the $V_{\text{max}}$ of glutamate oxidation measured in sonicates (78 nmol/min-mg). However, in the intact mitochondrial matrix, the presence of NADH and α-ketoglutarate prevents maximal activity of glutamate dehydrogenase. Data presented in Tables II and IV show that under most conditions only a small fraction of the glutamate which enters the mitochondria is metabolized to ammonia and α-ketoglutarate. Even when glutamate dehydrogenase is maximally stimulated in the presence of ADP and malonate at 28 °C, more than half of the entering glutamate is not metabolized. Since transport apparently becomes less rate limiting as the temperature is raised from 15 to 28 °C, it is likely that at 37 °C transport will present an even less severe limitation of flux through the dehydrogenase. This situation is quite different from that of kidney mitochondria where the $V_{\text{max}}$ of glutamate transport at 28 °C is 3.2 nmol/min-mg (42) and the $V_{\text{max}}$ of glutamate dehydrogenase measured under optimal conditions at 25 °C is 65 nmol/min-mg (24).

Secondly, the data show that α-ketoglutarate in the mitochondrial matrix can significantly regulate the rate of glutamate deamination (Fig. 8; Tables IV and V). It is likely that inhibition by matrix α-ketoglutarate has been the cause of the hitherto puzzling lack of activity of glutamate dehydrogenase in isolated mitochondria under coupled conditions in the presence of excess ADP (13). Inhibition by α-ketoglutarate provides a sensitive and versatile means of regulating ammonia formation in the intact cell since any effector which will inhibit glutamate transamination as uncoupler does would be expected to stimulate glutamate oxidation via the deamination pathway.

A carefully documented study of alanine metabolism in the liver has recently been published (9). The purpose of the study was to evaluate the relative importance of alanine transport across the plasma membrane and the glutamate dehydrogenase reaction as rate-controlling steps in the formation of glucose from alanine. Although the authors concluded that glutamate dehydrogenase is not rate limiting for alanine catabolism, it is apparent from their data that the ratio of mitochondrial glutamate/α-ketoglutarate correlates reasonably well with nitrogen flux, especially if one also takes into account changes in the mitochondrial redox state.

In the kidney, a decrease in pH has been shown to stimulate ammonia formation (43). This stimulation correlates with a fall in tissue α-ketoglutarate (44) and an increase in the rate of α-ketoglutarate oxidation, attributed to a H+ ion-induced decrease in the $K_m$ of α-ketoglutarate for the α-ketoglutarate dehydrogenase reaction (45). In view of the potent inhibitory effect of α-ketoglutarate on glutamine deamination, it is not surprising that stimulation of α-ketoglutarate oxidation has a dramatic influence on the rate of ammonia formation in the kidney. The control properties of α-ketoglutarate dehydrogenase are currently being investigated in a number of laboratories and demonstrated regulation by H+ and Ca2+ ions (46-48) provides intriguing possibilities for feedback regulation of glutamate dehydrogenase.

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**REFERENCES**

Ammonia Formation in Liver Mitochondria

Ammonia formation in isolated rat liver mitochondria.
K F LaNoue, A C Schoolwerth and A J Pease


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