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Quantitative Characteristics of Glutamate Transport in Rat Liver Mitochondria

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1. The kinetics of glutamate transport into mitochondria were determined by using Bromocresol Purple to terminate the transport process. 2. Glutamate transport was found to have a $V_{max.}$ of 9.1 nmol/min per mg of protein at pH 6.9 and 20°C; the K_m for glutamate was 4 mM. 3. The rate of glutamate deamination in intact mitochondria was tenfold slower than in disrupted mitochondria. 4. These results suggest that glutamate deamination may be controlled by the rate of glutamate transport. Possible consequences of these findings are discussed.

Two transporting systems for L-glutamate have been postulated to exist in the inner membrane of rat liver mitochondria (Azzi *et al.*, 1967). One carrier catalyses an electroneutral exchange between glutamate and hydroxyl ions and is inhibited by *N*-ethylmaleimide (Meijer *et al.*, 1972). The other carrier catalyses an obligatory coupled exchange between external glutamate and intramitochondrial aspartate. The mechanism of this carrier has not been fully elucidated.

The biochemical function of the glutamate/OH^- antiporter is thought to be the provision of glutamate for the production of NH_3 via glutamate dehydrogenase, which is exclusively located in the mitochondria. Glutamate entering on the glutamate/aspartate antiporter is necessarily transaminated with intramitochondrial oxaloacetate to form aspartate and is hence not available to the glutamate dehydrogenase. McGivan *et al.* (1973) have shown that leucine stimulates glutamate dehydrogenase in the deamination direction in disrupted, but not in intact, mitochondria. It was accordingly postulated that glutamate deamination in intact mitochondria is rate-limited by the transport of glutamate into the mitochondria. For this reason, it was decided to determine the kinetic characteristics of the glutamate/OH^- antiporter.

The 'inhibitor-stop' method originally devised for the determination of the kinetics of adenine nucleotide translocation (Pfaff & Klingenberg, 1968) has also been employed to study the kinetics of the transport of succinate (Quagliariello *et al.*, 1969), phosphate (J. D. McGivan & M. Klingenberg, unpublished work), citrate (Palmieri *et al.*, 1972*a*) and 2-oxoglutarate (Palmieri *et al.*, 1972*b*). It was not possible to apply this method to glutamate transport until an effective and rapidly acting inhibitor of glutamate transport had been found. In the experiments reported in the present paper it is shown that Bromocresol Purple is such an inhibitor, and this compound is used in the determination of the kinetics of glutamate transport by the 'inhibitor-stop' method.

Experimental

Rat liver mitochondria were isolated as described by Chappell & Hansford (1972). For the determination of the kinetics of glutamate transport, mitochondria (8–10 mg of protein/ml) were suspended in a medium containing 0.12 M-KCl, 0.02 M-Tris–HCl, 10 μM-rotenone, 10 μM-antimycin A, 0.1 mM-carboxymethoxylamine–HCl and ^3H_2O at the appropriate pH and temperature. [U-^{14}C]Glutamate was added, and glutamate uptake was terminated after a given time by the addition of a mixture of Bromocresol Purple (final concn. 1 mM) and *N*-ethylmaleimide (final concn. 0.15 mM). In parallel control experiments [U-^{14}C]glutamate was replaced by [U-^{14}C]-sucrose, which is a marker for the extra-mitochondrial space.

The mitochondrial suspension was then sedimented by centrifugation for 4 min at room temperature in an Eppendorf model 3200 microcentrifuge. The supernatant was removed and acidified with $HClO_4$ (final concn. 5%, w/v). The mitochondrial pellet was resuspended in 0.1 ml of water and deproteinized by the addition of $HClO_4$ (final concn. 5%, w/v). Samples of the acidified supernatant and of the acid extract of the pellet were assayed for ^{14}C and ^{3}H by dual-channel liquid-scintillation counting in a Packard Tri-Carb liquid-scintillation spectrometer, model 3380. The scintillator used contained in 1 litre: 600 ml of toluene, 400 ml of 2-methoxyethanol, 80 g of naphthalene and 6 g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole. It was found that the Bromocresol Purple extracted from the mitochondrial pellet did not interfere with the counting in this system. The matrix space of the mito-

chondria was assumed to be that available to ^3H_2O but not to [U-^{14}C]sucrose. The amount of glutamate taken up by the mitochondria was calculated by correcting the total [^{14}C]glutamate in the mitochondrial pellet for the ^{14}C contained in the extra-mitochondrial pellet water.

Citrulline was assayed by the method of Archibald (1944). Aspartate (Pfleiderer, 1963), glutamate (Bernt & Bergmeyer, 1963) and NH_3 (Kirsten *et al.*, 1963) were assayed enzymically. Mitochondrial protein was determined by a biuret method (Gornall *et al.*, 1949).

L-[U-^{14}C]glutamate, [U-^{14}C]sucrose and ^3H_2O were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Inhibition of glutamate transport by Bromocresol Purple

To determine the kinetic parameters of glutamate entry into mitochondria, it was necessary to find a compound that inhibited glutamate transport completely and very rapidly. The fact that Bromocresol Purple is an inhibitor of glutaminase (Sayre & Roberts, 1958) suggested that this substance might also inhibit other reactions involving glutamate. Fig. 1 shows that 0.1mM-Bromocresol Purple completely inhibited the swelling of rat liver mitochondria in iso-osmotic ammonium glutamate. Further experiments indicated that 0.1mM-Bromocresol Purple also inhibited mitochondrial respiration with glutamate as substrate.

Glutamate transport in liver mitochondria is also inhibited by *N*-ethylmaleimide (Meijer *et al.*, 1972). The effects of both of these compounds on the penetration of [U-^{14}C]glutamate into mitochondria are shown in Table 1. When the inhibitors were present before addition of glutamate, complete inhibition of

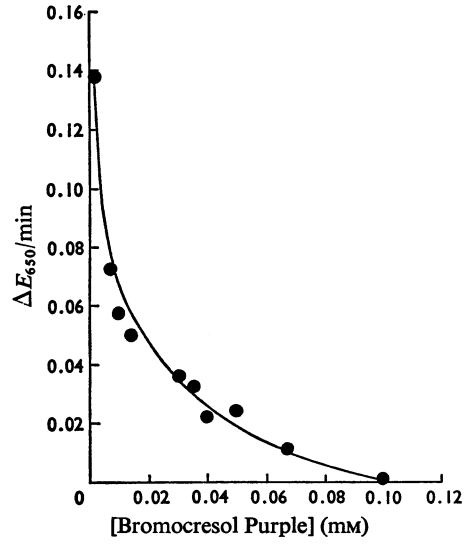

Fig. 1. *Inhibition of mitochondrial swelling in ammonium glutamate by Bromocresol Purple*

Mitochondria (1.5mg) were added to 2ml of a medium containing 0.1M-ammonium glutamate, 5μM-rotenone and 5μM-antimycin A at pH7.2 and 20°C. The initial rate of decrease of apparent absorbance owing to mitochondrial swelling was followed at 650nm.

Table 1. *Inhibition of glutamate uptake by Bromocresol Purple and N-ethylmaleimide*

The incubation medium was that described in the Experimental section at 20°C and pH7.0. Glutamate was added at 1mM final concentration. Bromocresol Purple (1mM) and *N*-ethylmaleimide (0.15mM) were present where indicated. Experimental error in the values is approx. 10%.

Experimental conditions	Final intramitochondrial concentration of glutamate (mM)		
Inhibitors added ...	Bromocresol Purple	*N*-ethylmaleimide	Bromocresol Purple + *N*-ethylmaleimide
Inhibitors added first, glutamate added 2min later	0.06	0.17	<0.02
Inhibitors and glutamate added simultaneously	0.10	0.69	<0.02
Glutamate added, followed by inhibitors after 30s. Immediate centrifugation	0.49	1.04	0.47
Glutamate added, followed by inhibitors after 30s. Centrifugation 3min later	0.44	1.60	0.46

glutamate entry occurred only when both inhibitors were present, although Bromocresol Purple when added alone gave a large inhibition. *N*-Ethylmaleimide was relatively ineffective. The rapidity with which the inhibition occurred was assessed by adding the inhibitors simultaneously with the glutamate. In this case also complete inhibition occurred only when both inhibitors were present. When glutamate was added first and the transport process was subsequently terminated by the addition of Bromocresol Purple plus *N*-ethylmaleimide, the amount of glutamate found in the pellet was independent of the amount of time that elapsed before centrifugation of the mitochondria.

It was concluded that a mixture of Bromocresol Purple plus *N*-ethylmaleimide inhibited glutamate transport sufficiently rapidly and completely to allow the determination of the parameters of glutamate transport by the 'inhibitor-stop' method.

Kinetics of glutamate uptake by rat liver mitochondria

The uptake of [U-^{14}C]glutamate was studied in mitochondria whose respiration was inhibited by the addition of rotenone plus antimycin A. Carboxymethoxylamine was also added to inhibit the intramitochondrial aspartate aminotransferase. Under these conditions, no aspartate could be formed. The amount of endogenous aspartate in mitochondria was found to be extremely low. Thus the uptake of glutamate represented only the activity of the glutamate/OH^- antiporter. In fact, the omission of carboxymethoxylamine did not alter the rate of glutamate uptake.

The time-course of glutamate uptake at 20°C is shown in Fig. 2(*a*). The uptake showed apparent first-order kinetics (Fig. 2*b*) similar to the transport of adenine nucleotides (Pfaff *et al.*, 1969) and succinate (Quagliariello *et al.*, 1969). Under the conditions used, the first-order rate constant was 0.62 min^{-1} and the rate of glutamate uptake was 2.7 nmol/min per mg of protein.

The concentration dependence of glutamate transport is indicated in Fig. 3(*a*). A Lineweaver–Burk plot (Fig. 3*b*) showed that glutamate transport had an apparent K_m for glutamate of 4 mM with a $V_{max.}$ of 9.1 nmol/min per mg at pH 6.9 and 20°C.

The rate of glutamate transport was faster at acid pH than at neutral pH (Fig. 4) and was relatively slow above pH 7. Similar pH dependences have been found for the transport of succinate (Quagliariello *et al.*, 1969) and phosphate (J. D. McGivan & M. Klingenberg, unpublished results) and may in part reflect a limitation of the transport process under these conditions by the external concentration of H^+ ions (which are effectively transported with the anionic species).

The rate of glutamate uptake increased less than

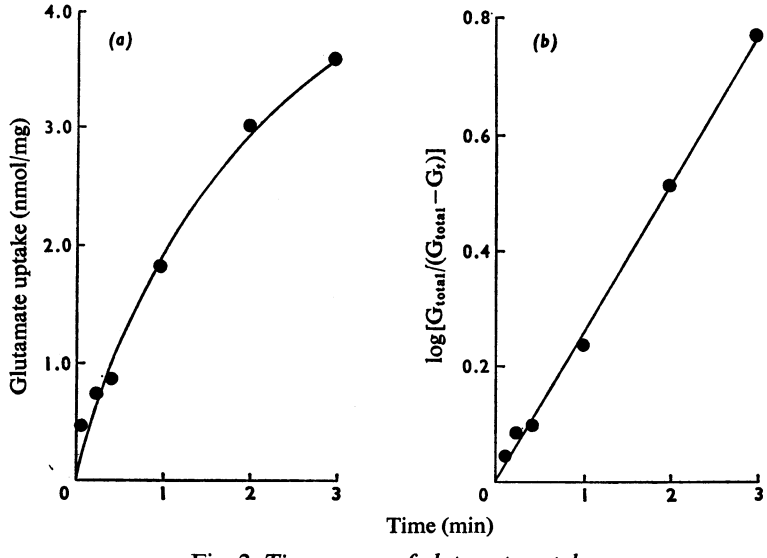

Fig. 2. *Time-course of glutamate uptake*

Glutamate uptake (*a*) was determined as described in the Experimental section. Glutamate was added at 1 mM concentration. The pH was 6.7 and the temperature 20°C. For the plot in Fig. 2(*b*) the total uptake of glutamate at equilibrium was assumed to be 4.5 nmol/mg. G_{total}, Total glutamate uptake at equilibrium; G_t, glutamate uptake at time *t*.

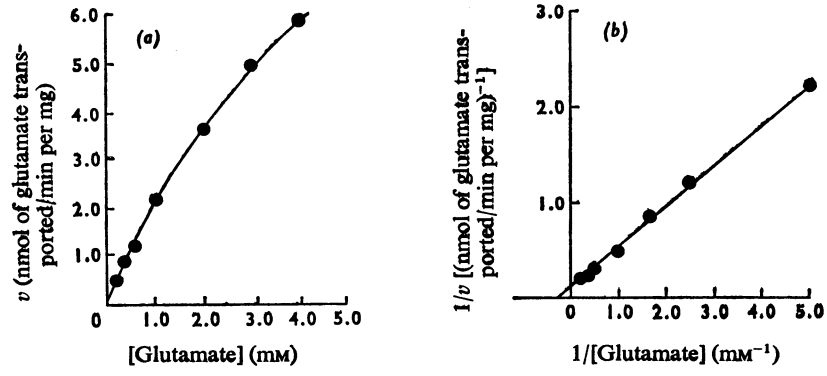

Fig. 3. *Concentration-dependence of glutamate uptake*

The experiment was performed as described in the Experimental section at pH 6.9 and 20°C. (*a*) Concentration dependence of glutamate transport and (*b*) Lineweaver–Burk plot of glutamate transport. For further details see the text.

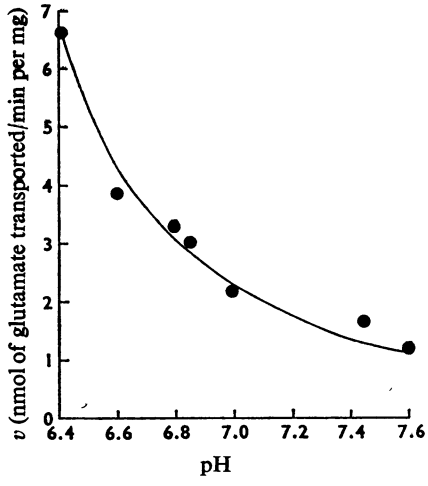

Fig. 4. *pH-dependence of glutamate uptake*

The glutamate concentration was 1 mM and the temperature was 20°C. For details see the Experimental section.

threefold between 5° and 20°C (Fig. 5*a*). An Arrhenius plot (Fig. 5*b*) indicated that the activation energy of glutamate transport was 36.9 J/mol (8.8 kcal/mol). This value is much less than those obtained for the other anion-translocating systems in liver mitochondria.

In some experiments the radioactive compounds appearing in the mitochondria were separated by small-column chromatography and identified. In each case, the intramitochondrial radioactivity appeared only in glutamate and 2-oxoglutarate. The ratio of these metabolites depended on the initial redox state of the intramitochondrial pyridine nucleotides before the [^{14}C]glutamate addition. This finding is consistent with initial penetration of glutamate as an intact molecule followed by equilibration via intramitochondrial glutamate dehydrogenase, which could not be inhibited in these experiments.

Reactions involving the deamination of glutamate

Glutamate dehydrogenase was assayed in the deamination direction in intact mitochondria by measurement of intramitochondrial NAD(P)$^+$ reduction on the addition of glutamate to mitochondria that had been preincubated with carbonyl cyanide phenylhydrazone to cause oxidation of the internal nucleotides and whose respiration was then inhibited by the addition of antimycin A. The rate of NAD(P)$^+$ reduction, which was due solely to glutamate dehydrogenase under these conditions, was relatively slow with an apparent $V_{max.}$ of 4.5 nmol/min per mg at pH 7.1 and 24°C and a K_m for glutamate of 2.4 mM (Fig. 6*a*). These parameters are similar to those observed for glutamate transport.

Glutamate dehydrogenase was assayed in sonicated mitochondria where no permeability barriers between enzyme and substrate exist. The dependence of the rate of reduction of NAD$^+$ in this system on glutamate concentration is shown in Fig. 6*b*. The $V_{max.}$ observed (66.7 nmol/min per mg) was more than ten times that measured in intact mitochondria under the same conditions of temperature and pH. The NAD$^+$ concentration used was 1 mM, which is similar to that occurring in intact mitochondria and was well above the K_m of the enzyme for NAD$^+$.

In isolated liver mitochondria, citrulline may be synthesized from a source of NH_3 in the presence of ornithine, bicarbonate and ATP (Charles *et al.*, 1967). When 10mM-glutamate was used as the NH_3 source, the rate of citrulline synthesis under optimum conditions at pH7.0 and 20°C was found to be

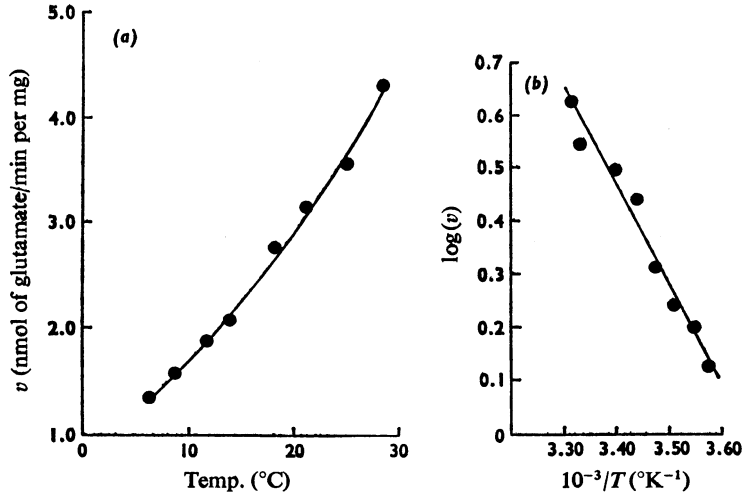

Fig. 5. *Temperature-dependence of glutamate uptake*

The glutamate concentration was 1mM and the pH was 6.9. For details see Experimental section.

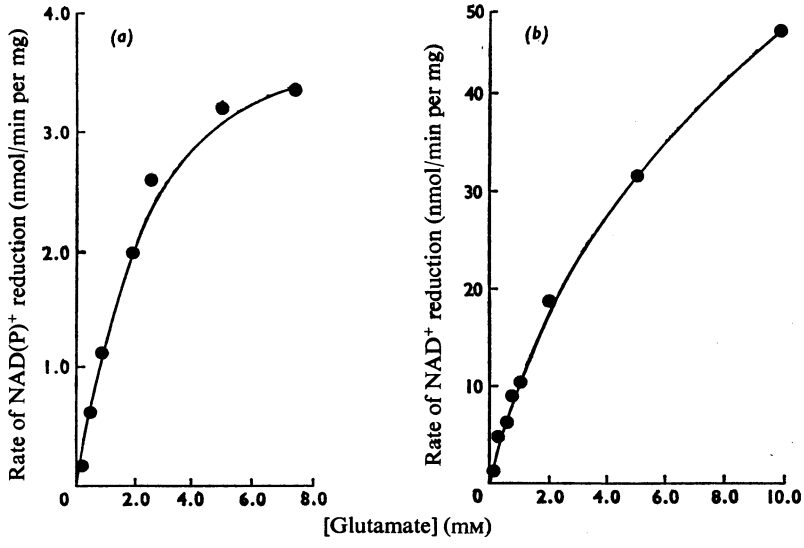

Fig. 6. *Activity of glutamate dehydrogenase in intact and disrupted mitochondria*

In (*a*), mitochondria (5mg/ml) were suspended in a medium containing 0.12M-KCl, 0.02M-Tris–HCl, 2mM-potassium phosphate and 1mM-sodium arsenite (to inhibit 2-oxoglutarate dehydrogenase) at pH7.1 and 24°C. Carbonyl cyanide phenylhydrazone (1μM) was added, followed after 3min by antimycin A (5μM). $NAD(P)^+$ reduction on the addition of glutamate was followed at 340–373nm by using a double-beam spectrophotometer. In (*b*), ultrasonically disrupted mitochondria (2 mg of protein/ml) were suspended in a medium containing 0.12M-KCl, 0.02M-Tris–HCl, 1mM-sodium arsenite and 10μM-rotenone at pH7.1 and 24°C. 1mM-NAD^+ was added and the initial rate of NAD^+ reduction on subsequent addition of glutamate was followed at 340nm.

2.0nmol/min per mg. Under the same conditions with 1mM-NH_4Cl as the NH_3 source, the rate of citrulline synthesis was 3–4 times higher. These results are consistent with the possibility that the rate-limiting step in the synthesis of citrulline from glutamate as NH_3 source is the transport of glutamate across the mitochondrial membrane, since this has been shown to be much slower than the maximum rate of glutamate dehydrogenase in sonicated mitochondria.

The rate of NH_3 formation from added glutamate by respiring mitochondria was determined under various conditions (Table 2). NH_3 production was relatively slow even when the formation of aspartate was inhibited by the addition of carboxymethoxylamine or of malonate. The rate of NH_3 production was never greater than the rate of glutamate transport observed in respiration-inhibited mitochondria at the same pH and temperature, suggesting again that glutamate deamination is limited by the activity of the glutamate/OH^- antiporter. When malate was present, the rate of aspartate formation was relatively high. For aspartate formation to occur, glutamate must enter the mitochondria and transaminate with the intramitochondrial oxaloacetate produced by malate oxidation. The rate of aspartate formation was much greater than the activity of the glutamate/OH^- antiporter determined earlier. In this case, the glutamate could enter on the glutamate/aspartate exchange carrier, which apparently has a much higher activity than the glutamate/OH^- antiporter.

Discussion

The rate of glutamate uptake in the kinetic experiments presented above reflects only the activity of the glutamate/OH^- antiporter, since under the conditions used there was no possibility of aspartate formation. It is clear that the glutamate/OH^- antiporter has a very much lower activity than have the carrier systems for the other mitochondrial substrates. Table 3 summarizes the results obtained for a number of these transporting systems. At 9°C the rate of glutamate transport is an order of magnitude lower than the rates of transport of other metabolites. Since the activation energy of glutamate transport (36.9kJ/mol; 8.8kcal/mol) is also lower than that of the other anion translocations (in most cases 84–105kJ/mol; 20–25kcal/mol) it follows that the difference between the rates of transport of glutamate and other metabo-

Table 2. *NH_3 and aspartate formation during glutamate oxidation by rat liver mitochondria*

Mitochondria were suspended in a medium containing 0.12M-KCl, 20mM-Tris–HCl, 10mM-glucose, 2mM-$MgSO_4$, 5mM-potassium phosphate, 1mM-ADP and 20μg of dialysed hexokinase/ml at pH7.2 and 20°C. The reaction was started by the addition of 10mM-glutamate. Samples of the suspension were withdrawn after 3, 6 and 9min, deproteinized and assayed for NH_3 and aspartate.

Additions	Rate of formation of metabolites (nmol/min per mg)	
	NH_3	Aspartate
None	<0.1	2.5
Carbonyl cyanide phenylhydrazone (1μM)	0.6	1.3
Malonate (5mM)	1.7	<0.1
Carboxymethoxylamine (0.1mM)	1.3	<0.1
Malate (5mM)	<0.1	20

Table 3. *Kinetic constants of anion translocation in rat liver mitochondria*

Anion-transporting system	$V_{max.}$ at 9°C (nmol/min per mg)	K_m for named substrate (mM)	Reference
ADP/ATP	70	0.012	Pfaff *et al.* (1969)
Succinate	50	0.83	Quagliariello *et al.* (1969)
Phosphate	80	0.25	J. D. McGivan & M. Klingenberg (unpublished work)
2-Oxoglutarate	43	0.046	Palmieri *et al.* (1972*b*)
Citrate	23	0.09	Palmieri *et al.* (1972*a*)
Glutamate	5	4.0	The present paper

lites is much greater at 37°C than it is at 9°C. The relatively high K_m of the glutamate-transporting system for its substrate is a further factor suggesting that glutamate transport under conditions existing in the liver cell *in vivo* is relatively very slow. It should be emphasized that the glutamate/OH^- antiporter is the only mechanism for the supply of external glutamate to intramitochondrial glutamate dehydrogenase, since glutamate entering on the glutamate/aspartate carrier is necessarily transaminated to form aspartate.

It is of worth to consider whether the low activity of the glutamate/OH^- antiporter could be rate-limiting for any metabolic processes in the liver cell. Several lines of evidence indicate that in isolated mitochondria the rate of glutamate deamination is limited by the rate of glutamate transport. Thus glutamate deamination in intact mitochondria was not significantly stimulated by leucine although glutamate dehydrogenase in disrupted mitochondria was markedly stimulated (McGivan *et al.*, 1973). The apparent activity of glutamate dehydrogenase in the deamination in intact mitochondria was tenfold less than the activity of glutamate dehydrogenase in sonicated mitochondria (Fig. 6). The rate of citrulline synthesis from glutamate as NH_3 source was similar to the corresponding rate of glutamate transport, whereas NH_4Cl as an NH_3 source gave a much faster rate of citrulline synthesis. Finally, the rates of NH_3 production from externally added glutamate in respiring mitochondria were similar to the observed rates of glutamate transport (Table 2) and were much slower than the corresponding rates of glutamate deamination in sonicated mitochondria.

In the liver cell, most of the amino groups obtained by the catabolism of amino acids are transferred to 2-oxoglutarate in various transamination reactions with the formation of glutamate. The NH_3 required for the synthesis of carbamoyl phosphate and hence of urea is thought to arise partly from the deamination of glutamate (originally produced in the cytoplasm) by intramitochondrial glutamate dehydrogenase. The rate of synthesis of urea from alanine as N source in perfused rat liver at 37°C is approximately 0.34 μmol/min per g wet weight (Chalamaun & Tager, 1970). It can be estimated that this rate of urea production requires a rate of transport of glutamate across the mitochondrial membrane of 5–10 nmol/min per mg of mitochondrial protein. It is noteworthy that this value is of the same order of magnitude as the rates of glutamate transport into isolated mitochondria reported in the present paper.

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