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TRANSPORT OF GLUTAMATE IN RAT-LIVER MITOCHONDRIA

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SUMMARY

The transport of glutamate across the membrane of rat-liver mitochondria has been studied.

1. Glutamate can be transported across the mitochondrial membrane in exchange for OH^- (or together with H^+).
2. Intramitochondrial glutamate is not extruded from the mitochondria by addition of aspartate when the mitochondria are preloaded with glutamate.
3. *N*-Ethylmaleimide is a specific inhibitor of the movement of glutamate across the mitochondrial membrane.

INTRODUCTION

The inner membrane of rat-liver mitochondria contains a number of anion-specific translocators that bring about a 1:1 exchange between certain substrate–anion pairs[1-3].

In 1967 Azzi *et al.*[4] concluded that glutamate is able to enter the mitochondrial matrix in two ways: either *via* a separate glutamate translocator that can be specifically inhibited by certain analogues of glutamate, or *via* a separate aspartate translocator in exchange for aspartate.

In an earlier study[5], we obtained apparently anomalous results with respect to the mechanism of transport of glutamate. These results prompted the experiments described in this paper, which deals mainly with the mechanism by which glutamate is transported *via* the glutamate translocator[4].

METHODS

Rat-liver mitochondria were prepared acording to the method of Hogeboom[6] as described by Myers and Slater[7].

Loading of mitochondria with glutamate. The mitochondrial preparation (100–150

Abbreviations: DMO, 5,5′-dimethyloxazolidine-2,4-dione; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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mg protein) obtained from one liver was incubated at 0 °C in a medium containing 100 mM potassium glutamate, 100 mM sucrose, 10 mM Tris–HCl and 50 μg rotenone in a volume of 6 ml and at a pH of 7.0. After 2 min, 20 ml of an ice-cold solution containing 250 mM sucrose *plus* 10 mM Tris–HCl (pH 7.0) was added, following which the mitochondria were collected by centrifugation at 0 °C. The mitochondria were washed with 20 ml of a cold solution containing 250 mM sucrose, 10 mM Tris–HCl (pH 7.0) and 50 μg rotenone. After centrifugation, the mitochondria were finally taken up in 1–2 ml of the same solution and stored at 0 °C. Mitochondria prepared in this way rapidly release the glutamate accumulated into the suspension medium. It is therefore necessary to use these mitochondria as rapidly as possible after the loading. Corrections must be made for the leakage of glutamate that occurs during the course of the experiment.

Oxygen uptake was measured polarographically using a Clark-type electrode and the Gilson oxygraph.

Separation of the mitochondria from the suspension medium was done exactly as described earlier[8].

Radioactivity was measured as described by Harris and van Dam[9].

Assays. Glutamate, α-oxoglutarate, aspartate and malate were determined in neutralized perchloric acid extracts of the mitochondria and of the suspension medium by enzymic methods as described previously[5], using either a Zeiss PMQ 4 spectrophotometer (for the determination of glutamate) or the Aminco-Chance dual-wavelength spectrophotometer at the wavelength pair 350–375 nm.

Chemicals. Mersalyl was obtained from Sigma, *N*-ethylmaleimide from British Drug Houses, and valinomycin and nigericin from Eli Lilly and Company. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from E. J. Dupont De Nemours and Company. [^{14}C]Glutamate was obtained from the Radiochemical Centre (Amersham) and ^3H_2O from Philips Duphar (Netherlands).

RESULTS

The mechanism of glutamate transport across the mitochondrial membrane

Azzi *et al.*[4] have shown that respiration-inhibited rat-liver mitochondria rapidly swell in an isoosmotic solution of ammonium glutamate. Since it is likely that ammonia is transported across the mitochondrial membrane in the undissociated form[10] the conclusion must be that glutamate exchanges with OH^- (or is co-transported with a H^+).

In order to rule out the possibility that glutamate exchanges for intramitochondrial phosphate which, in turn, exchanges with OH^- *via* the phosphate translocator, the effect of mersalyl on the swelling process was investigated; this compound is known to be a strong inhibitor of the phosphate-translocating system[11]. Fig. 1 demonstrates that concentrations of mersalyl sufficiently high to prevent swelling of the mitochondria in isoosmotic ammonium phosphate have no effect on the swelling process in ammonium glutamate. Similar results were obtained with *p*-hydroxymercuribenzoate and 5,5′-dithio-bis-(2-nitrobenzoate), two other inhibitors of the phosphate translocator[12,13] (not shown). These results rule out a role of the phosphate translocator in ammonium glutamate-induced swelling.

If glutamate can exchange with OH^- (or be co-transported with H^+) in an analogous manner to the transport of phosphate on the phosphate translocator[10,14], one would expect that mitochondria can be made to swell in isoosmotic potassium glutamate, provided both valinomycin (for induction of K+ permeability[15]) and uncoupler (for induction of H+ permeability[16,17]) are present. We had previously observed[5] that extensive swelling occurred in the presence of valinomycin alone, and that this swelling was largely abolished upon the addition of uncoupler. This effect is also shown in Fig. 2B. Fig. 2A shows the control experiment with potassium phosphate, where both valinomycin and uncoupler are necessary to obtain a maximal rate of swelling (*cf.* refs 17, 18).

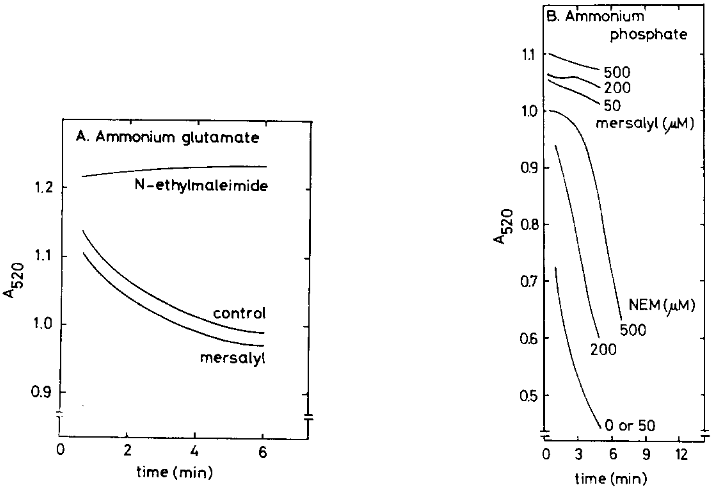

Fig. 1. Effect of *N*-ethylmaleimide and mersalyl on swelling of rat-liver mitochondria in isoosmotic ammonium glutamate and ammonium phosphate. Mitochondria (0.42 mg protein) were suspended in a medium containing 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 μg rotenone and 125 mM ammonium glutamate (A) or 100 mM ammonium phosphate (B). Where indicated, 300 μM *N*-ethylmaleimide (NEM) or 100 μM mersalyl were present. Volume, 2 ml; temperature, 20 °C.

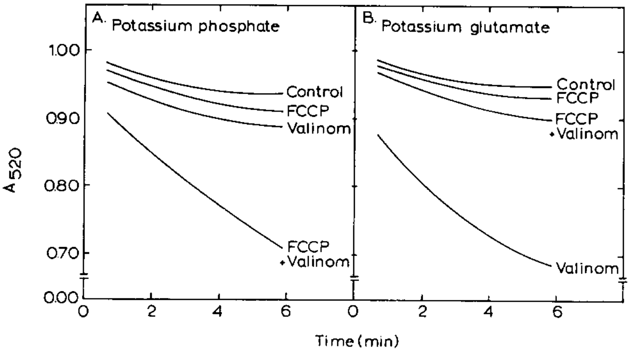

Fig. 2. Effect of valinomycin and FCCP on mitochondrial swelling in isoosmotic potassium phosphate or potassium glutamate. Mitochondria (0.35 mg protein) were incubated as described in Fig. 1 except that the potassium salts of phosphate and glutamate were used. Where indicated, 0.01 μg valinomycin and 1 μM FCCP were present.

The observation that valinomycin alone induces maximal mitochondrial swelling in a potassium glutamate solution under these conditions suggested that the glutamate anion is able to pass the mitochondrial membrane as such (*cf.* thiocyanate[18]). On the other hand it was difficult to understand why an uncoupler should inhibit this process. A closer investigation of the system revealed that a leak in the rotenone block is probably responsible for these phenomena. Table I shows that despite the presence of rotenone there was some metabolism of the glutamate added. This can be concluded from the formation of both α-oxoglutarate and malate (formation of aspartate could not be detected). This result suggests that the induction of swelling by valinomycin under these conditions may be energy dependent. One possibility is that an energy-dependent uptake of K^+ in exchange for H^+ occurs, followed by a glutamate–hydroxyl exchange. This process will, of course be sensitive to uncoupler. The leak in the rotenone block may be sufficient to support the energy-linked uptake of K^+ if one takes into account that at least 2 $K^+/\sim$ can be transported during respiration[19]. The energy dependence is considered further in the Discussion.

In Table II the effect of valinomycin, uncoupler and nigericin on the efflux of glutamate from the mitochondria was studied. In the presence of the uncoupler FCCP alone, a considerable extrusion of glutamate from the mitochondria occurred. The extrusion increased with increasing uncoupler concentration; this effect is

TABLE I

METABOLISM OF GLUTAMATE IN THE PRESENCE OF ROTENONE

For studying the metabolism of glutamate, mitochondria (6.5 mg protein) were incubated in a volume of 1 ml for 4 min exactly as described in the legend to Fig. 2B. The absorption measurement was carried out in a volume of 2 ml, using 0.3 mg mitochondrial protein.

Additions	*ΔMalate (nmoles)*	*Δα-Oxoglutarate (nmoles)*	*ΔA_{520}/4 min*
None	9	14	0.038
Valinomycin (0.02 μg)	17	14	0.103
FCCP (1 μM)	43	29	0.040
Valinomycin + FCCP	43	28	0.076

TABLE II

EFFLUX OF GLUTAMATE FROM THE MITOCHONDRIA

Reaction medium: 65 mM Tris–HCl (pH 7.0), 2 mM EDTA, 5 mM $MgCl_2$, 1% alcohol, 2 μg rotenone, and mitochondria (7.1 mg protein) preloaded with glutamate (see Methods). Final volume, 1 ml; temperature, 20 °C; reaction time, 1 min.

Additions	*$Glutamate_{in}$ (nmoles)*	*$Glutamate_{out}$ (nmoles)*	*$Glutamate_{total}$ (nmoles)*
None	78	256	334
Valinomycin (0.1 μg)	59	266	325
FCCP (0.03 μM)	57	261	318
FCCP (0.10 μM)	36	289	325
Valinomycin + FCCP (0.03 μM)	40	282	322
Valinomycin + FCCP (0.10 μM)	18	308	326
Nigericin (0.1 μg)	31	293	324

analogous to the effect of uncoupler on intramitochondrial phosphate[20]. Valinomycin alone also caused some extrusion of glutamate. The effects of FCCP and valinomycin were purely additive. Nigericin, which catalyses a 1:1 exchange between K^+ and H^+ (ref. 21), was much more effective than valinomycin in causing an extrusion of glutamate from the mitochondria. Thus these results, too, indicate that glutamate can cross the mitochondrial membrane in exchange for OH^- (or together with H^+).

More evidence in favour of a glutamate–hydroxyl exchange was obtained from the experiment of Table III, where the effect of various anions on the oxidation of succinate in the presence of Ca^{2+} was investigated. The rationale of this experiment is that when an anion can exchange with OH^- (or can be transported together with H^+) it must be able to release the inhibition of respiration in State 6. From Table III it can be seen that phosphate, acetate and 5,5′-dimethyloxazolidine-2,4-dione (DMO), which are typical representatives of such anions[10,22], did, indeed, stimulate respiration under these conditions. It is of interest that both acetate and DMO have their optimal effects at pH 6.5. This behaviour may be due to the fact that the concentration of the undissociated acids is highest at low pH values. In contrast, phosphate exerts its maximum effect at pH 7.5. The reason for this optimum is not known; perhaps the activity of the phosphate translocator has a pH optimum near 7.5.

TABLE III

EFFECT OF VARIOUS ANIONS ON THE OXIDATION OF SUCCINATE IN THE PRESENCE OF Ca^{2+}

Mitochondria (2.9 mg protein) were incubated in a medium containing 100 mM Tris–HCl, 10 mM succinate, 2 mM $CaCl_2$ and 2 μg rotenone. After 1 min, the potassium salts of the anions indicated in the table were added at a final concentration of 10 mM. Temperature, 25 °C; final volume, 1.5 ml.

Anion added	*Oxygen consumption (natoms O/mg per min) at*		
	pH 6.5	*pH 7.5*	*pH 8.5*
Chloride	57	45	26
Phosphate	235	401	86
Acetate	194	135	55
DMO	217	146	74
Thiocyanate	63	48	36
Glutamate	102	54	37

Thiocyanate, which is transported exclusively as the anion[18] had hardly any effect on State 6 respiration at all three pH values tested. Finally, glutamate had little or no effect at pH 7.5 or 8.5, and caused a significant stimulation of respiration at pH 6.5. The behaviour at pH 6.5 indicates that the glutamate anion can be exchanged for OH^- (or be transported together with H^+). Since the glutamate translocator has its optimal activity below pH 6 (ref. 4) it is not surprising that the ability of glutamate to release State 6 respiration is greater at pH 6.5 than at pH 7.5 or 8.5.

The inhibition of glutamate transport by N-ethylmaleimide

In 1969 Haugaard *et al.*[13] reported that oxidation of glutamate in uncoupled rat-liver mitochondria is blocked by *N*-ethylmaleimide. We have confirmed this observation. Since high concentrations of mersalyl or 5,5′-dithio-bis-(2-nitrobenzoate) have no effect under these conditions[5,13] it may be concluded that the inhibition by *N*-ethylmaleimide is not related to the activity of the phosphate translocator. The following experimental evidence shows that the inhibitory effect of *N*-ethylmaleimide on the uncoupled oxidation of glutamate can be ascribed to an inhibition of glutamate translocation across the mitochondrial membrane.

First, Fig. 1A shows that *N*-ethylmaleimide completely prevented swelling of mitochondria in an isoosmotic solution of ammonium glutamate. Mersalyl, on the other hand, had no effect.

Second, the uptake of [^{14}C]glutamate by the mitochondria was inhibited by *N*-ethylmaleimide, but not by mersalyl (Table IV). It should be noted that the radioactivity measurement in Table IV represents not only radioactive glutamate, but also presumably some α-oxoglutarate and malate (*cf.* Table I).

Thirdly, the leakage of glutamate from mitochondria preloaded with glutamate was prevented almost completely by *N*-ethylmaleimide, but not by mersalyl (see Fig. 3). It should be noted that the concentration of mersalyl applied was much higher

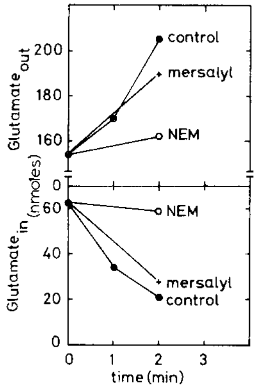

Fig. 3. Effect of *N*-ethylmaleimide and mersalyl on the efflux of glutamate from mitochondria. Mitochondria (4.5 mg protein) preloaded with glutamate were incubated in a medium containing 15 mM KCl, 50 mM Tris–HCl (pH 7.0), 2 mM EDTA, 5 mM $MgCl_2$, 2 μg rotenone and (where indicated) 600 μM *N*-ethylmaleimide (NEM) or 400 μM mersalyl. Volume, 1 ml; temperature, 20 °C.

TABLE IV

EFFECT OF *N*-ETHYLMALEIMIDE AND MERSALYL ON THE ACCUMULATION OF GLUTAMATE BY RAT-LIVER MITOCHONDRIA

Conditions: Mitochondria (6.7 mg protein) were incubated in a medium (1 ml) containing 15 mM KCl, 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, ^3H_2O, 3 μg rotenone and (where indicated) 600 μM *N*-ethylmaleimide or 600 μM mersalyl. After 2 min, 1 mM [^{14}C]glutamate was added; 45 s later the incubation was terminated by centrifugation of the mitochondria. Temperature, 20 °C. The intramitochondrial glutamate concentrations were corrected for glutamate present in the sucrose space. The values given in the table are the means of triplicate incubations.

Addition	*Glutamate$_{in}$* (*mM*)
None	2.40
N-Ethylmaleimide	1.22
Mersalyl	2.59

than that needed to inhibit either the phosphate translocator or the dicarboxylate translocator[23].

Inability of extramitochondrial aspartate to exchange with intramitochondrial glutamate

Since glutamate can pass the mitochondrial membrane in two ways, either *via* the glutamate translocator (presumably in exchange from OH^-; see above) or *via* the aspartate translocator in exchange for aspartate[4], it was of interest to investigate if this last process is sensitive to *N*-ethylmaleimide. We therefore loaded mitochondria with glutamate and tried to cause extrusion of the latter by adding aspartate to the medium. Table V shows that in the presence of rotenone, aspartate was unable to induce any significant extrusion of the glutamate from the mitochondria. Since LaNoue and Bryła[24] have reported that the efflux of aspartate is energy dependent, the mitochondrial energy state was varied. However, under no conditions could an aspartate-induced extrusion of glutamate be observed (Table V). It is noteworthy that addition of aspartate always caused a small increase in the total amount of glutamate present in the system. This must have been due to transamination of aspartate with endogenous α-oxoglutarate, which may have been derived from glutamate (*cf.* Table I).

TABLE V

INABILITY OF ASPARTATE TO EXCHANGE WITH INTRAMITOCHONDRIAL GLUTAMATE

Reaction conditions: Mitochondria (7.5 mg protein) preloaded with glutamate were incubated in a medium containing 15 mM KCl, 50 mM Tris–HCl (pH 7.0), 2 mM EDTA, 5 mM $MgCl_2$, 2 µg rotenone and (where indicated) 10 mM aspartate, 1 µM FCCP, 3 mM ATP or 5 mM succinate. Final volume, 1 ml; temperature, 20 °C; incubation time, 1 min.

Additions	*Glutamate$_{in}$ (nmoles)*	*Glutamate$_{out}$ (nmoles)*	*Glutamate$_{total}$ (nmoles)*
None	71	177	248
Aspartate	85	190	275
FCCP	27	230	257
FCCP + aspartate	28	247	275
ATP	60	182	242
ATP + aspartate	68	189	257
Succinate	87	156	243
Succinate + aspartate	87	176	263

DISCUSSION

Our experiments suggest that glutamate can be transported across the mitochondrial membrane in exchange for hydroxyl ions (or together with H^+)*. This is supported by the following evidence: (a) swelling of the mitochondria in isoosmotic ammonium glutamate[4]; (b) the effects of valinomycin, FCCP and nigericin on glutamate efflux from the mitochondria; (c) the ability of glutamate to release inhibition of respiration in State 6.

The apparent anomalous behaviour of the mitochondria in an isoosmotic solution of potassium glutamate in the presence of valinomycin can possibly be

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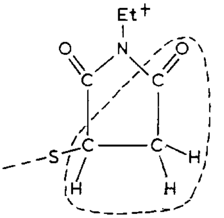
* It should be stressed that the exchange of glutamate for hydroxyl (or transport of glutamate together with a proton) refers to the end situation. The transport of the hydroxyl (or proton) may be indirect, *via* an ionizing group of the translocator or of the substrate.

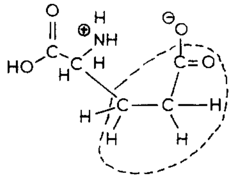
accounted for at first sight by an energy-linked accumulation of K^+ due to a small leak in the rotenone block. However, not only swelling in potassium glutamate (+ valinomycin) but also that in ammonium glutamate is inhibited by uncoupler (not shown). This observation suggests that the energy requirement is not specifically restricted to the uptake of K^+. Our results do not allow us to distinguish between an energy requirement for cation uptake or an effect of energization on the transport of the glutamate anion.

The following observations suggest that *N*-ethylmaleimide is an inhibitor of glutamate transport across the mitochondrial membrane: (a) *N*-ethylmaleimide inhibits glutamate oxidation in uncoupled mitochondria[13]; (b) it inhibits mitochondrial swelling in isoosmotic ammonium glutamate; (c) it inhibits the uptake of [^{14}C]glutamate; (d) it inhibits leakage of glutamate from mitochondria. Of the various -SH-binding reagents tested, *N*-ethylmaleimide is the only one that inhibits glutamate translocation[5].

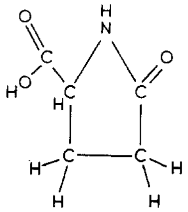
It is possible that the specific inhibition by *N*-ethylmaleimide is due to the formation of a substrate analogue when *N*-ethylmaleimide is bound to a thiol group:



N-Ethylmaleimide (bound)



Glutamate



Pyrrolidone-2-carboxylate

The structural analogy between glutamate and thiol-bound *N*-ethylmaleimide would be even more striking if a lactam is formed from glutamate. In this connection it is of interest to note that pyrrolidone-2-carboxylic acid has been postulated as an intermediate in the mechanism of transport of amino acids in rat kidney[25].

Inability to demonstrate an aspartate–glutamate exchange

Azzi *et al.*[4] proposed that aspartate is transported across the mitochondrial membrane in exchange for glutamate. This was based on the observation that extramitochondrial aspartate could not readily react with intramitochondrial aspartate transaminase without glutamate being present. The experiment of Table V shows that extramitochondrial aspartate is *not* able to extrude glutamate from the mitochondria under the experimental conditions used. A possible explanation for this phenomenon may be found in the suggestion of McGivan and Chappell (cited in ref. 2) that aspartate is not transported as such but as oxaloacetate *via* membrane-bound aspartate transaminase. According to this mechanism, aspartate is not able to cross the mitochondrial membrane unless α-oxoglutarate is present to transaminate with it. If this is so, then extramitochondrial aspartate can only cause extrusion of that intramitochondrial glutamate which originates from the transamination reaction between extramitochondrial aspartate and α-oxoglutarate. This mechanism could explain why extramitochondrial aspartate is not able to cause an extrusion of glutamate from mitochondria preloaded with glutamate.

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