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KINETIC STUDY OF GLUTAMATE TRANSPORT IN RAT LIVER MITOCHONDRIA

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SUMMARY

The transport of glutamate across the inner membrane of rat liver mitochondria has been studied by the swelling technique and by direct measurement of [^{14}C]-glutamate penetration.

1. Glutamate transport is inhibited by uncharged liposoluble –SH reagents such as fuscin, *N*-ethylmaleimide and avenaciolide which are able to reach the matrix; it is not inhibited by ionic –SH reagents such as mersalyl. Thus, the –SH groups involved in the transport of glutamate are located in a hydrophobic part of the membrane or on the matrix side of the membrane.

2. The kinetic study of glutamate penetration reveals saturation kinetics and a high temperature and pH dependence. The results are the following: (a) pH_{opt}, 6.5; (b) K_m, 5 mM and V, 23 nmoles/min per mg protein at 25 °C and pH 6.5, (c) E_A, 17.5 kcal/mole at pH 6.5.

3. Compared to the Krebs cycle anion transporting systems, the glutamate transporter has high K_m and low V values; this seems to limit the role of glutamate as a carrier of reducing equivalents into the mitochondria. The implication of the kinetics of glutamate uptake in urea synthesis is discussed.

INTRODUCTION

It is well established that the inner membrane of rat liver mitochondria is permeable to glutamate[1]. It has been shown[2] that this amino acid enters the mitochondrial matrix together with a proton or in exchange for an OH^-. Some evidence has been presented[1] that glutamate can also be exchanged for aspartate. The existence of an additional glutamate (or aspartate)–α-ketoglutarate exchange has been suggested[3]. A wide range of glutamate transport inhibitors have been described so far, including glutamate analogs[1], *N*-ethylmaleimide[2], avenaciolide[4], fuscin[5,6], tannic acid and fluorodinitrobenzene[7].

In this paper we first compare the effects of some inhibitors, namely fuscin, *N*-ethylmaleimide, avenaciolide and mersalyl, on mitochondrial swelling in isotonic solutions of ammonium glutamate and ammonium phosphate. We then report some kinetic properties of glutamate penetration in rat liver mitochondria.

METHODS

Rat liver mitochondria were prepared by the method of Hogeboom[8]. Rats were fasted 14–16 h before sacrifice. When mitochondria were prepared for swelling experiments 1 mM EDTA was added to the standard sucrose medium. Mitochondrial proteins were assayed by the biuret method.

Swelling experiments

The absorbance of mitochondrial suspensions was recorded at 520 nm on a Cary 15 spectrophotometer. For particular conditions, see legends of the figures.

Penetration of [^{14}C]glutamate

The reaction was initiated by adding mitochondria to the incubation medium in 1-ml Eppendorf cups (see specific conditions in the legends). After time t the mitochondria were separated from the incubation medium by rapid centrifugation in an Eppendorf 3200 microcentrifuge. It was not necessary to stop the uptake with an inhibitor, since the time course is linear for 30 s, and the centrifugation time lag is only 10 s (see Fig 5). The supernatant fluid was discarded and the tightly packed pellet was quickly rinsed with 1 ml of ice-cold 100 mM KCl solution. The pellet was dissolved in 0.5 ml of a 4 % sodium cholate solution. The radioactivity of the cholate extracts was measured in an Intertechnique SL 30 scintillation counter in the POPOP/PPO system. Corrections for glutamate present in the sucrose space were made as follows in a parallel series of tubes [^{14}C]sucrose or ^3H_2O was added instead of [^{14}C]glutamate[9], and the radioactivity of the pellets was measured as described for glutamate. From the radioactivity of the pellet containing [^{14}C]sucrose we could determine the extramatrix space (permeable to sucrose), and from the pellet containing ^3H_2O the total water space (permeable to ^3H_2O). The total water space has also been determined by gravimetry; the values found by this method were identical to those of the ^3H_2O space According to the experimental conditions the matrix space ranged from 0.6 to 0.8 μl/mg protein, whereas the sucrose space varied from 1.5 to 2.2 μl/mg protein.

Chemicals

Mersalyl, *N*-ethylmaleimide, rotenone, oligomycin, antimycin A, L-glutamic acid, D-glutamic acid, γ-ethyl and γ-methyl esters of L-glutamic acid were purchased from Sigma, L-γ-hydroxyglutamic acid and dithiothreitol (Cleland's reagent) were bought from Calbiochem, Los Angeles Calif., U.S.A., [U-^{14}C]sucrose and ^3H_2O were obtained from the "Commissariat à l'Energie Atomique", Paris, France. L-[U-^{14}C]glutamic acid was bought from the Radiochemical Center, Amersham, Bucks (U.K).

Fuscin was a generous gift of Dr D H. R. Barton (Imperial College of Science and Technology, London). Samples of avenaciolide and analogs were kindly provided by Dr W. B. Turner (I.C I Ltd, Macclesfield, U K.).

RESULTS AND DISCUSSION

Comparative study of the inhibitory properties of fuscin, N-ethylmaleimide, avenaciolide and mersalyl

Mitochondrial swelling in ammonium salts of permeant anionic substrates is due to net substrate accumulation; thus, in the case of phosphate or glutamate, since no other permeant anion is present in the medium, only the phosphate–hydroxyl or glutamate–hydroxyl exchanges, respectively, can account for mitochondrial swelling. Consequently, in the swelling experiments shown hereafter, the eventual interference of the glutamate–aspartate or glutamate–ketoglutarate exchanges is ruled out.

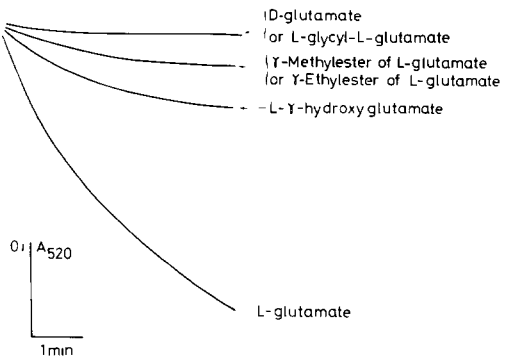

Fig. 1. Swelling of rat liver mitochondria in ammonium salts of L-glutamate and analogs. Rat liver mitochondria (1.4 mg) were added at time zero to 2 ml of a medium containing 100 mM ammonium L-glutamate or analog, 20 mM Tris, 1 mM EDTA, 10 μM rotenone. The pH was adjusted at 7.5. Temperature was 25 °C. The absorbance was recorded at 520 nm.

Fig. 1 shows that the glutamate–OH^- carrier is very specific for L-glutamate. King and Diwan[7] have shown that ^{14}C-labelled D-glutamate is unable to penetrate into rat liver mitochondria; this is confirmed here by the fact that rat liver mitochondria do not swell at all in an isotonic solution of ammonium D-glutamate. The dipeptide L-glycyl-L-glutamate is also non penetrant; other analogs of L-glutamate, namely L-γ-hydroxyglutamate, γ-methyl or ethyl esters of L-glutamate are hardly more penetrant.

Fig. 2 shows the effects of fuscin, *N*-ethylmaleimide, avenaciolide, and mersalyl on mitochondrial swelling in ammonium phosphate and ammonium glutamate. The corresponding quantitative results are given in Table I. Fuscin and *N*-ethylmaleimide inhibit both phosphate and glutamate penetration, but are much more effective on the latter. Avenaciolide, the most specific and effective inhibitor of glutamate transport, has no effect at all on phosphate penetration. As shown by Meijer *et al.*[2], mersalyl does not inhibit glutamate entry. Glutamate analogs at a concentration of 5 mM have no effect at all on glutamate penetration; this absence of inhibition might be due to the high glutamate concentration (100 mM) compared to that of the analogs.

It is worth noticing that fuscin, *N*-ethylmaleimide, and avenaciolide inhibit

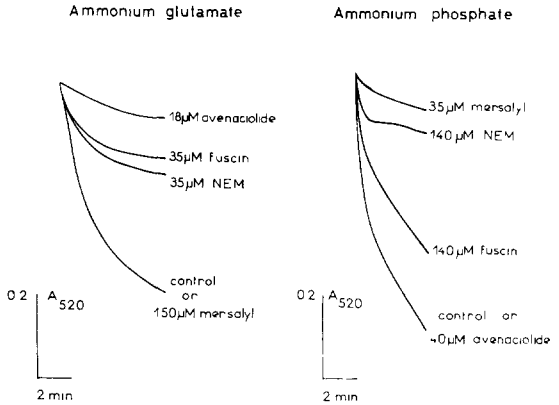

Fig 2. Inhibition of mitochondrial swelling in ammonium glutamate or phosphate Rat liver mitochondria (1 2 mg) were added at time zero to 2 ml of the following medium 100 mM ammonium glutamate or phosphate, 20 mM Tris, 1 mM EDTA, 10 μM rotenone pH was 7.5 and temperature 25 °C Where indicated the inhibitors were present in the medium before the addition of mitochondria

glutamate penetration in the same range of concentrations (20–50 nmoles/mg protein). As shown on Fig. 3 all these inhibitors possess a carbon–carbon double bond (arrow) activated by a conjugated carbonyl double bond. Thiols can readily bind on this double bond of *N*-ethylmaleimide[10] fuscin[11], and avenaciolide[12]. This common chemical property of the three inhibitors is confirmed by the experiment described in Fig. 4, where, for the sake of clarity, only the results with avenaciolide are shown, the same being obtained with *N*-ethylmaleimide and fuscin. When dithiothreitol is

TABLE I

CONCENTRATIONS OF INHIBITOR (nmoles/mg PROTEIN) NECESSARY TO REACH A 50 % INHIBITION OF MITOCHONDRIA SWELLING

The conditions were the same as in Fig. 2 Note that there is no preincubation of the mitochondria with the inhibitors. After a preincubation of 2–3 min at room temperature a 50 % inhibition would be obtained at a lower concentration of inhibitor

Inhibitor	*Swelling in ammonium glutamate*	*Swelling in ammonium phosphate*
Mersalyl	No inhibition*	10
Fuscin	45	200
N-Ethylmaleimide	55	150
Avenaciolide	20	No inhibition**
Iso-avenaciolide	100	No inhibition**
Ethisolide	No inhibition***	No inhibition***
D-Glutamate or L-γ-Hydroxyglutamate	No inhibition****	No inhibition****

* Up to 500 nmoles/mg protein.
** Up to 60 nmoles/mg protein
*** Up to 150 nmoles/mg protein.
**** Up to 5000 nmoles/mg protein.

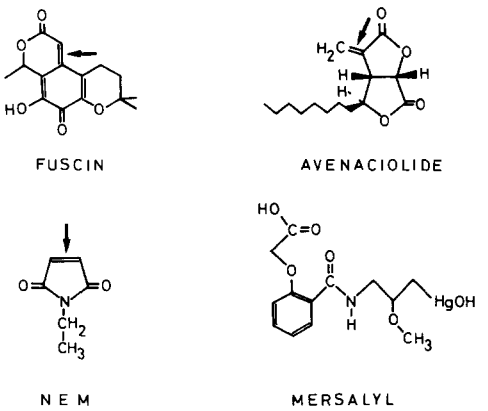

Fig. 3. Structures of fuscin, avenaciolide, *N*-ethylmaleimide and mersalyl. The reactive double bonds of fuscin, avenaciolide and *N*-ethylmaleimide are shown by arrows

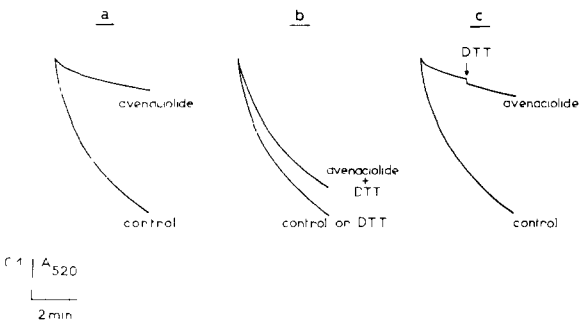

Fig 4. Mitochondrial swelling in ammonium glutamate, inhibition by avenaciolide and prevention of inhibition by dithiothreitol (DDT) The conditions were the same as in Fig. 2 Rat liver mitochondria (1 9 mg) were added at time zero. Avenaciolide was present as indicated at a concentration of 25 μM. Dithiothreitol was used at a concentration of 0.5 mM. In Fig 4b avenaciolide and dithiothreitol were added to the medium 1 minute before the mitochondria The same results were obtained with *N*-ethylmaleimide and fuscin (not shown here)

added to the glutamate and inhibitor containing medium before the mitochondria (Fig. 4b), no inhibition is observed, this shows that the inhibitor (*N*-ethylmaleimide, fuscin, or avenaciolide) has reacted with dithiothreitol. However, the inhibition is not reversed if dithiothreitol is added after the mitochondria (Fig. 4c) This irreversibility of the inhibition points to a covalent binding of the inhibitor to thiol groups It is also remarkable that *N*-ethylmaleimide, fuscin, and avenaciolide are uncharged species, and quite soluble in a hydrophobic phase. On the other hand, mersalyl is a hydrophilic charged compound, and it inhibits the phosphate–OH^- but not the glutamate–OH^- exchange From these results we can conclude that the thiol group(s) of the phosphate–OH^- carrier is (are) probably located in an outer and hydrophilic part of the membrane (accessible to mersalyl), whereas the SH group(s) of the glutamate–OH^- carrier is located in a hydrophobic part of the membrane (inaccessible to mersalyl and accessible to *N*-ethylmaleimide, fuscin and avenaciolide)

Avenaciolide[4] and *N*-ethylmaleimide[2] have been supposed to inhibit glutamate transport because of a structural analogy with glutamate. This is not supported by our results, nor by the chemical evidence; these inhibitors, like fuscin, seem to function as SH reagents and have been shown to react with intramitochondrial glutathione[6].

Aldridge and Turner[13] have isolated two analogs of avenaciolide iso-avenaciolide, which differs from avenaciolide by the orientation of the eight carbon aliphatic side chain, and ethisolide, which differs from iso-avenaciolide by the replacement of the eight carbon side chain by an ethyl group.

Iso-avenaciolide is about five times less effective than avenaciolide, and ethisolide does not inhibit glutamate transport at all (Table I). This suggests that the side chain of avenaciolide might help to plug the molecule into a lipophilic part of the membrane and thus place the methylene group in a position favouring its reaction with a thiol of the glutamate–OH^- carrier

Kinetic study of [^{14}C]glutamate penetration in rat liver mitochondria

When one measures the penetration of [^{14}C]glutamate into mitochondria, one generally uses glutamate concentrations of 10^{-2} to 10^{-4} M In these conditions, unlike in swelling experiments, the possible exchanges with intramitochondrial substrates cannot be neglected. Glutamate can penetrate either on the glutamate–OH^- carrier, or on the glutamate–aspartate carrier, or even on an hypothetical glutamate–α-ketoglutarate carrier. Meijer *et al.*[2] have tried to overcome this difficulty by loading mitochondria with high amounts of glutamate and measuring its efflux from the mitochondrial matrix. We have studied the direct penetration of [^{14}C]glutamate and determined some of its kinetic parameters.

Time course of glutamate uptake The time course of glutamate penetration is shown on Fig. 5. The curve is linear for about 30 s at 25 °C; at lower temperatures the linear portion is even longer (40 to 60 s) The extrapolation of the initial straight line towards the time axis shows that the actual incubation time is 10 s longer than the

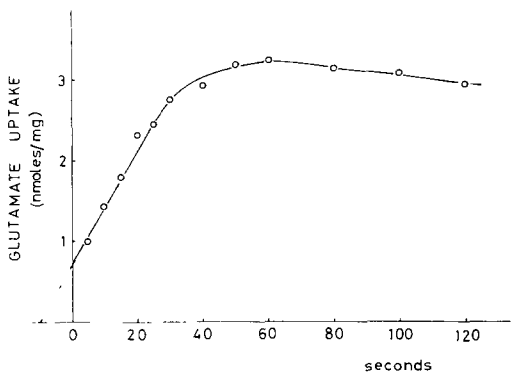

Fig 5 Time course of L-[^{14}C]glutamate penetration in rat liver mitochondria. Mitochondria (4 8 mg) were added to 0 95 ml of a medium containing 100 mM KCl, 10 mM 2-(*N*-morpholino)-ethanesulphonic acid, 10 mM Tris, 20 μM rotenone, 2 5 μg oligomycin, 1 mM [^{14}C]glutamate pH was 6 5 and temperature 25 C For other conditions, see Methods

measured one: this is so because glutamate penetration goes on during the first seconds of centrifugation, before most of the mitochondria are gathered in the pellet (the reaction is not stopped by addition of an inhibitor, but by flash centrifugation, see Methods). The amount of glutamate accumulated in the matrix reaches a maximum after 60 s, and then decreases slightly. This might be due to a leakage of glutamate, or to the exit of some of its metabolites.

Saturation kinetics Fig. 6 shows that glutamate penetration in rat liver mitochondria follows saturation kinetics. The K_m is 5 mM at 25 °C and pH 6.5. This value is much higher than those reported for other mitochondrial anion transports[14,15,3]. However, it is of the order of magnitude of the physiological concentration of glutamate in liver[16,17], and might be in agreement with a possible regulatory role of glutamate transport. The Lineweaver–Burk plot of Fig. 6 yields only one straight line, but this does not imply that only one carrier is involved in glutamate penetration. Christensen[18] has shown that, to be discretely recognizable, two K_m values must be separated from each other by at least one order of magnitude. The value for maximum velocity is 23 nmoles/min per mg of protein at 25 °C and pH 6 5 It is significantly lower than the V for other anion transports[14,15,3]. However, it is sufficient to account for the maximum velocity of oxygen consumption by rat liver mitochondria with glutamate as substrate. Furthermore, the rate of glutamate uptake is high enough to feed the urea cycle, even if glutamate were the only ammonium source for the mitochondrial synthesis of carbamyl phosphate; indeed, urea biosynthesis requires less than 10 nmoles of intra-mitochondria ammonia/min per mg protein at 37 °C (ref. 19), whereas the velocity of glutamate uptake at the same temperature, extrapolated from Fig. 8 (glutamate concentration was 1 mM), is about 12 nmoles/min/mg protein

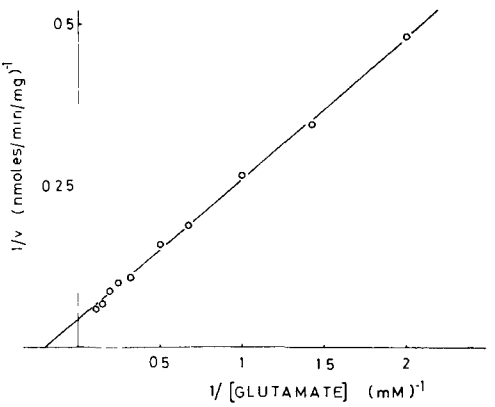

Fig. 6. Lineweaver–Burk plot of [^{14}C]glutamate penetration in rat liver mitochondria Same conditions as in Fig. 5, except mitochondria 4 mg, glutamate concentration was varied from 0 5 mM to 9 mM, and incubation time was 30 s

pH effect. As shown on Fig. 7, glutamate uptake is strongly pH dependent. Like most of the mitochondrial anion transports, it is favoured by acid pH, with a maximum between pH 6 and 6.5. The ionic state of glutamate probably contributes

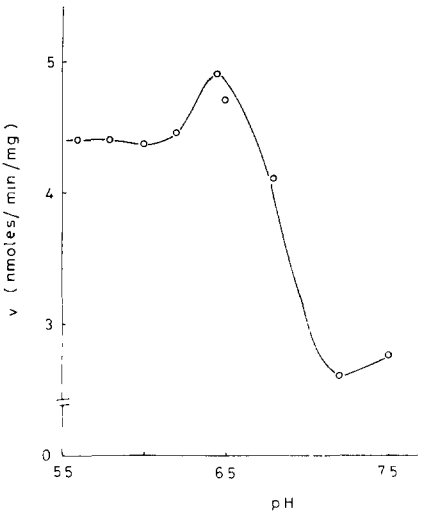

Fig. 7 Effect of pH on [^{14}C]glutamate penetration in rat liver mitochondria Conditions as in Fig 5, except that 4 mg mitochondria were added to the medium, incubation time was 30 s

very little to this pH effect, since the p*K*s of this amino-acid (2, 4 and 9) are very distant from the optimum pH value for transport.

Temperature effect. The temperature dependence of glutamate transport is shown on Fig. 8. The Arrhenius plot is a straight line. The activation energy calculated from the slope is 17.5 kcal/mole of glutamate; this value is close to those found for other anion transports[14,15,3]. The temperature interval used here is 8–28 °C. Below 8 °C the penetration of glutamate is too slow and the initial velocity of uptake cannot be measured accurately; it is thus impossible to determine whether there is or not a temperature transition in the region of 8 °C to 12 C. Such a transition has been evidenced in the adenine-nucleotide exchange[20,21]

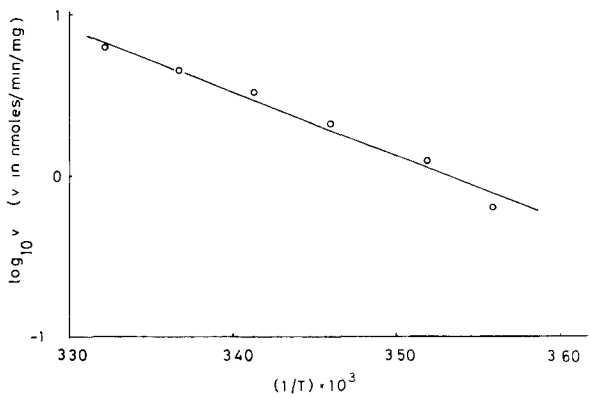

Fig 8 Temperature dependence of [^{14}C]glutamate penetration in rat liver mitochondria Conditions as in Fig 5. Mitochondria 3 4 mg, incubation time 30 s

CONCLUSIONS

Using the swelling technique (swelling of mitochondria in ammonium glutamate) (refs 2, 4 and this paper) a whole range of inhibitors of the glutamate–OH^- carrier can be evidenced, however, the effects of these inhibitors on the glutamate–aspartate[1] and glutamate–glutamine exchange[22] are unknown. In our experiments on [^{14}C]glutamate penetration we have thus been unable to discriminate between the different glutamate carrier systems that have been postulated so far. However, the kinetics of glutamate show some interesting properties. First, the K_m value is much higher than those of other mitochondrial anion transporting systems; this must be correlated to the high glutamate concentration in the liver tissue, which is of the same order of magnitude as the K_m value. The second characteristic feature is the low velocity of glutamate uptake compared to those of other mitochondrial anion transporters; this is a strong limitation to the role of glutamate as a carrier of reducing equivalents into the mitochondrion. The main role of glutamate might well be to feed the mitochondrial matrix with ammonia. Indeed, as we have shown, glutamate uptake is rapid enough to provide intramitochondrial ammonia for carbamyl phosphate synthesis, the velocities of glutamate uptake and of urea synthesis are of the same order of magnitude. Thus, the values of K_m and V suggest that glutamate transport across the mitochondrial membrane might have a regulatory function in the physiology of the liver cell.

The pH and temperature effects on glutamate transport do not differ significantly from those of other mitochondrial anion transporters, and deserve no further comment

NOTE ADDED IN PROOF (Received October 31st, 1973)

The data presented in this paper are in good agreement with those, just published, of Bradford, N M. and McGivan, J. D. (1973) *Biochem. J.* 134, 1023–1029.

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