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# FUMARATE PERMEATION IN RAT LIVER MITOCHONDRIA: FUMARATE/MALATE AND FUMARATE/PHOSPHATE TRANSLOCATORS

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SUMMARY Fumarate permeation in isolated rat liver mitochondria was demonstrated by measuring malate and phosphate efflux caused by fumarate added externally to the mitochondrial suspension. The existence of two specific fumarate translocators, fumarate/malate and fumarate/phosphate, is shown here. These carriers are distinguished in the light of different kinetic parameters (Km values are 50 μM and 150 μM, and Vmax values are 17 and 40 nmoles/min x mg mitochondrial protein, respectively) and of differing sensitivity to non-penetrant compounds. Fumarate was found to cause oxaloacetate efflux from mitochondria by means of an indirect process which involves the cooperation of both fumarate/malate and malate/oxaloacetate translocators.
Results are discussed in the light of the physiological role played by fumarate translocation in both ureogenesis and aminoacid metabolism. 

The overall pathway of urea formation involves enzyme reactions in both cytosol and mitochondria, thus necessitating transport of intermediates across the mitochondrial membrane (Scheme 1). Ornithine and citrulline translocation has been shown previously (1-3). Fumarate was initially reported to be a non penetrant anion (4) not inhibiting malonate, citrate and oxoglutarate uptake into RLM *in vitro* (5). Neither was mention made of fumarate permeation in subsequent investigations concerning the role of anion translocation in urea synthesis as well as interrelationships between gluconeogenesis and ureogenesis in isolated hepatocytes (6,7). Nonetheless, our own studies, which demonstrate the occurrence of carrier-mediated translocation for fumarate in RHM, RKM and RBM (8-10) along with the recent finding that only 20% of the total fumarase

Abbreviations : AA, aminoacids; ARG, arginine; ARGSUCC, arginin-succinate; ASP, aspartate; Carb-Pi, carbamyl-phosphate; CTRL, citrulline; EGTA, ethylene -bis(oxyethylenenitrilo)tetraacetic acid; FUM, fumarate; GABA, γ-amino-butyrate; GLUT, glutamate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MAL, malate; MDH, malate dehydrogenase; NEM, N-ethyl-maleimide; OAA, oxaloacetate; ORN, ornithine; PEP, P-enolpyruvate; PEPcK, P-enolpyruvate carboxykinase; Pi, inorganic phosphate; PYR, pyruvate; RBM, rat brain mitochondria; RHM, rat heart mitochondria; RKM, rat kidney mitochondria; RLM, rat liver mitochondria.

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activity in rat liver is present in the cytosol (11), prompted us to reinvestigate fumarate permeation in RLM. The existence is shown in this paper of two separate carriers for fumarate in RLM, fumarate/malate and fumarate/phosphate translocators.

MATERIALS & METHODS All reagents used were from SIGMA (St. Louis, U.S.A.), except [$^{14}$C]-fumarate which was from Radiochemical Center (Amersham, England).
Rat liver mitochondria were isolated according to (12) with mitochondrial protein determined by the Waddel and Hill method (13).
Phosphate efflux was monitored by measuring intramitochondrial inorganic phosphate according to (14) ($\varepsilon_{735}$ was 21 $mM^{-1}cm^{-1}$ at 20°C).
The percentage exchange was calculated according to the equation: percentage exchange = 100 (Pa-Pp)/Pa, where Pa and Pp respectively represent the nmoles of phosphate measured in the absence and presence of external anion.
Measurements of [$^{14}$C]-fumarate were carried out by using the inhibitor stop method, essentially according to (15); radioactivity in the mitochondrial pellet was measured as described (16).
Malate efflux induced by extramitochondrially added anions was monitored as previously reported (17), by adding malic enzyme and $NADP^+$ outside mitochondria and following $NADP^+$ reduction. Changes in the redox state of $NADP^+$ were followed either photometrically or fluorimetrically using a Beckmam DU-7 HS spectrophotometer and a Perkin-Elmer LS-5 luminometer respectively. In the latter case, in order to obtain quantitative measurements of $NADP^+$ reduction rate, calibration was made of the fluorimetric response, according to (18). Modification was made of the cuvette holder to allow continuous stirring of each sample and addition of substrates without opening the cover.
Fumarate/OAA and malate/OAA exchanges were monitored essentially according to (19) by adding MDH and NADH outside mitochondria and following NADH oxidation caused by externally added anion.
The rates of change of fluorescence and absorbance were obtained as tangent at the initial part of the experimental curve and expressed as nmoles $NADP^+$ reduced (or NADH oxidized)/min x mg mitochondrial protein.

RESULTS

In the phase outside mitochondria, malate concentration can be considered negligible, since no change in fluorescence occurs following malic enzyme (0.2 e.u.) addition to RLM in the presence of $NADP^+$ (0.25 mM). Further addition of fumarate (0.2 mM), which has no effect when added alone, produces an extensive reduction of the pyridine nucleotide (Fig. 1). Thiocyanate (10 mM), a fumarase non-competitive inhibitor (20) able to enter mitochondria (21), completely inhibits $NADP^+$ reduction rate. 32 % and 54 % inhibition was found when fumarate was added in the presence of either 0.2 mM butylmalonate, inhibitor of the dicarboxylate carrier, or 0.1 mM N-ethylmaleimide, inhibitor of Pi carrier respectively (for ref. see 22).

One possible explanation for these findings could be that fumarate enters mitochondria in exchange with endogenous malate or phosphate (see below). Once inside the matrix, fumarate is hydrated to malate which in turn leaves mitochondria in exchange for further fumarate (Fig. 2A). However, the possible cooperation of two different

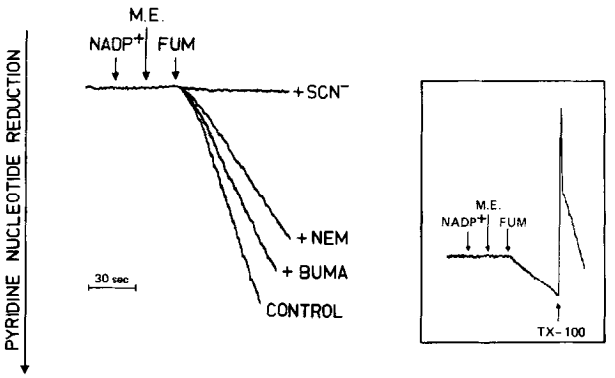



Fig. 1 Appearance of malate in the incubation medium induced by the addition of fumarate to rat liver mitochondria.
RLM (1.4 mg protein) were preincubated at 20°C for 1 min in 2.0 ml of standard medium containing 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES-Tris pH 7.20 plus 2 µg rotenone. Where indicated, additions were as follows: 0.25 mM $NADP^+$, 0.2 units of malic enzyme (M.E.) (previously dialyzed against 100 mM Tris-HCl buffer at pH 7.0), 0.2 mM fumarate (FUM), 10 mM thiocyanate ($SCN^-$), 0.1 mM N-ethyl-maleimide (NEM), 0.2 mM butylmalonate (BUMA) and 0.5 % Triton X-100 (T X-100).
$NADP^+$ reduction was followed fluorimetrically.

carriers, namely a carrier able to mediate fumarate/Pi exchange and the dicarboxylate carrier, in causing malate efflux cannot be excluded at this stage (Fig. 2B).

The addition of Triton X-100 (0.5 %) (see inset Fig. 1) to mitochondria during $NADP^+$ reduction, following a rapid change of fluorescence due to mitochondria dissolution, increases the $NADP^+$ reduction rate about 4 times, thus showing that the rate of fumarate transport in mitochondria is the limiting step in the measured rate of fluorescence increase. Controls indicate that Triton does not change the activity of either malic enzyme or intramitochondrial fumarase.

A further experiment (Table 1) carried out under the same experimental conditions shows the ability of externally added fumarate (0.1 mM) to cause direct Pi efflux from the mitochondrial matrix (Fig. 2C). Thiocyanate, malic enzyme and $NADP^+$ were present in each sample to inhibit fumarase and remove any possible malate effluxed from mitochondria in exchange with fumarate, thus ruling out the possibility that Pi efflux could occur by means of both fumarate/malate and dicarboxylate carriers (Fig. 2D). Neither butylmalonate (0.2 mM) nor the thiol reagents, mersalyl (0.18 mM) and N-ethylmaleimide (0.1 mM), powerful inhibitors of the Pi carrier (see 22), effect Pi efflux, whereas the substrate analogue methylfumarate (10 mM) strongly prevented Pi efflux. As a control, the ability of malate to cause Pi efflux via dicarboxylate

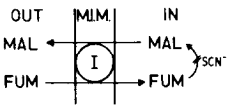
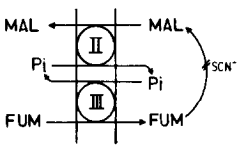
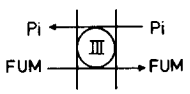
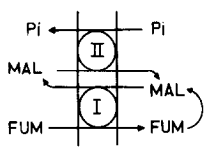
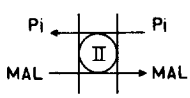
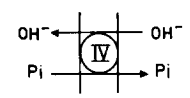
| MECHANISM FOR FUMARATE TRANSPORT | SENSITIVITY TO DIFFERENT COMPOUNDS | | |
|---|---|---|---|
| | METHYLFUMARATE | NETHYLMALEIMIDE | BUTYLMALONATE |
| A   | + (THIS PAPER) | + (THIS PAPER) | + (THIS PAPER) |
| B   | + | - | + |
| C   | + (THIS PAPER) | - (THIS PAPER) | - (THIS PAPER) |
| D   | + | + | + |
| E   | - (REF. 5) | - (REF. 15 and THIS PAPER) | + (REF. 22 and THIS PAPER) |
| F   | - (REF. 5) | + (REF. 22) | - (REF. 22) |

Fig. 2 Mechanism of fumarate transport into rat liver mitochondria.
I Fumarate/malate carrier; II Dicarboxylate carrier; III Fumarate/phosphate carrier; IV Phosphate carrier.
+ and - indicate the presence or absence of inhibition by the tested compounds; when + or - are squared the result was experimentally found; on the other hand, they indicate the inhibition to be theoretically found in the case of occurrence of the proposed mechanism, when they are circuited.

carrier was measured in the same experiment in the absence or presence of the same inhibitors. Malate/Pi exchange was found to be insensitive to both methylfumarate and NEM, but inhibited by both butylmalonate and mersalyl.

The inhibitor sensitivity, summarized in Fig. 2, clearly show that mechanisms described in A and C are in line with experimental results, whereas other possibilities (B-D-E-F) are not consistent with experimental data.

TABLE I

EXCHANGE OF INTRAMITOCHONDRIAL PHOSPHATE WITH EXTERNALLY ADDED COMPOUNDS

| ADDITIONS | INTRAMITOCHONDRIAL PHOSPHATE (nmoles/mg protein) | EXCHANGE (%) | CONTROL (%) |
|---|---|---|---|
| NONE | 26.5 | 0 | |
| FUMARATE | 18.3 | 31 | 100 |
| FUMARATE + METHYLFUMARATE | 23.6 | 11 | 35 |
| FUMARATE + BUTYLMALONATE | 17.9 | 32 | 103 |
| FUMARATE + NEM | 18.6 | 30 | 97 |
| FUMARATE + MERSALYL | 18.1 | 32 | 103 |
| MALATE | 12.1 | 54 | 100 |
| MALATE + METHYLFUMARATE | 12.9 | 51 | 94 |
| MALATE + BUTYLMALONATE | 19.3 | 27 | 50 |
| MALATE + NEM | 12.4 | 53 | 98 |
| MALATE + MERSALYL | 16.1 | 39 | 72 |

Mitochondria (1.2 mg protein) were incubated at 20°C for 1 min in 1.0 ml of the standard medium. In the case of fumarate 0.25 mM $NADP^+$, 0.2 units of malic enzyme, 10 mM thiocyanate were also present.
The exchange reaction was started by rapid addition of the metabolites (100 µM) and stopped 1 min later by rapid addition of methylfumarate (30 mM) (as regard fumarate) and butylmalonate (20 mM) (as regard malate), and by centrifuging with an Heraeus microcentrifuge. Where indicated the inhibitors, methylfumarate (10 mM), butylmalonate (10 mM), N-ethylmaleimide (NEM) (0.1 mM) and mersalyl (0.18 mM), were added along with fumarate or malate.
% of exchange was calculated as reported in Methods.

Fumarate permeation in isolated mitochondria has been also investigated by measuring mitochondrial swelling. In agreement with Chappel and Haarhoff (4), mitochondria were found to swell very slightly in isotonic ammonium fumarate solutions. However very low amplitude swelling can be measured which is more evident if compared with the constancy of absorbance of mitochondria suspended in either isotonic sucrose or ammonium chloride solutions (not shown).

In order to confirm fumarate permeation in RLM, [$^{14}$C]-fumarate uptake was investigated directly according to the stop inhibitor method, using 20 mM butylmalonate as an inhibitor (not shown). It should be noted that, owing to the activity of the intramitochondrial enzymes, pellet radioactivity could be due not only to fumarate, but also to malate and succinate and other derivated metabolites formed in the matrix. The effect of several compounds (10 mM each) on fumarate (50 µM) permeation was also tested. While very strong inhibition of fumarate uptake and

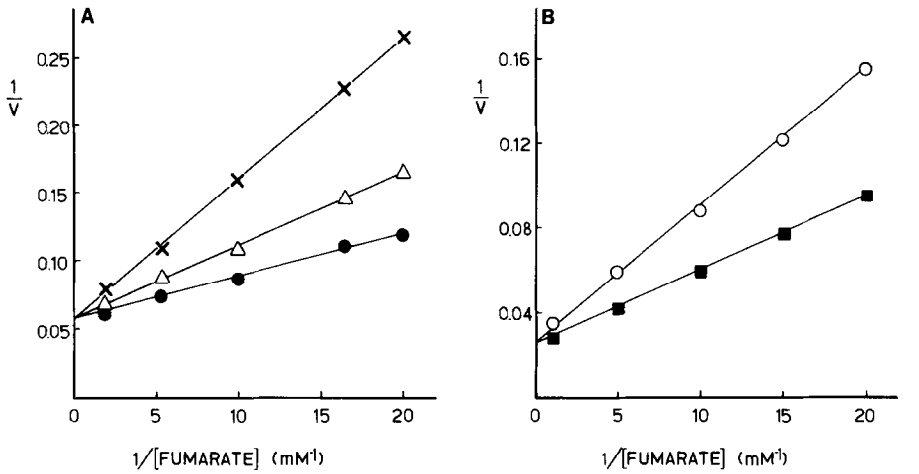



Fig. 3 Kinetic analysis of the inhibition of both fumarate/malate and fumarate/phosphate exchanges by phenylsuccinate or butylmalonate and methylfumarate respectively, using the double reciprocal plot.
RLM (1.5 and 1.2 mg protein in A and B, respectively) were preincubated at 20°C in 2.0 ml and 1.0 ml of standard medium in the absence (A) or in presence of 0.25 mM NADP+, 0.2 units of malic enzyme and 10 mM thiocyanate (B), respectively.
A. The experiment was carried out as described in Fig. 1. Fumarate was added at the indicated concentrations in the absence (●) or in presence of 1 mM phenylsuccinate (Δ) and 0.2 mM butylmalonate (X). The rate V is expressed as nmoles NADH oxidized/min x mg mitochondrial protein.
B. The exchange reaction was started by rapid addition of fumarate, at the indicated concentrations, in the absence (■) or presence of 10 mM methylfumarate (○), and terminated 15 sec later by rapid addition of methylfumarate (30 mM) and by centrifuging with an Heraeus microcentrifuge. The rate V is expressed as nmoles phosphate effluxed/min x mg mitochondrial protein.

metabolism was caused by phosphate and by the impermeable inhibitors butylmalonate and maleate, no significant change in radioactivity taken up was found to be caused by either OAA or citrate.

In order to gain further insight into fumarate uptake and metabolism, use was made in this paper of both photometric or fluorimetric techniques.

The dependence of both fumarate/malate and fumarate/Pi exchange rate on increasing fumarate concentrations was tested both in the absence and presence of either 0.2 mM butylmalonate or 1 mM phenylsuccinate, and 10 mM methylfumarate, respectively. Saturation characteristics were found in typical experiments (Fig. 3). In the first case (Fig. 3A) the Km, i.e. fumarate concentration giving half maximum NADP+ reduction rate, and Vmax values were found to be 50 μM and 17 nmoles NADP+ reduced/min x mg protein. Both butylmalonate and phenylsuccinate, non-penetrant inhibitors of both dicarboxylate and oxodicarboxylate carriers in RLM, RHM and RKM (5,15,17,23,24), and OAA and GABA carriers in RBM (19,25) give competitive inhibition,

with Ki values respectively equal to 0.08 mM and 1.2 mM (Fig. 3A). On the contrary, these compounds have no effect on the rate of fumarate/Pi exchange (Km and Vmax values were 150 μM and 40 nmoles Pi effluxed/min x mg mitochondrial protein), which was competitively inhibited by methylfumarate (Ki = 14.5 mM) (Fig. 3B). In the latter case, the initial rate of fumarate/Pi exchange was measured by stopping the reaction with 30 mM methylfumarate.

Possible fumarate translocation via the dicarboxylate, tricarboxylate and oxodicarboxylate carriers had been previously excluded owing to the lack of fumarate and methylfumarate inhibition on malonate, citrate, oxoglutarate and oxaloacetate uptake into RLM (5,26).

In another experiment, fumarate and malate were compared in terms of their ability to cause intramitochondrial OAA efflux, measured according to (19). The Triton experiment showed that the measured rate of fluorescence increase depends on the rate of translocation across the mitochondrial membrane in the cases of fumarate/OAA and malate/OAA as well. The double reciprocal plot for fumarate/OAA and malate/OAA exchanges showed the occurrence of saturation characteristics with the same value of Vmax statistically evaluated and different Km values (Vmax = 14.8 nmoles NADH oxidized/min x mg protein in the reported experiment; Km = 1.2 and 2.2 mM for fumarate and malate respectively) (Fig. 4). Thus, malate and fumarate apparently share the same

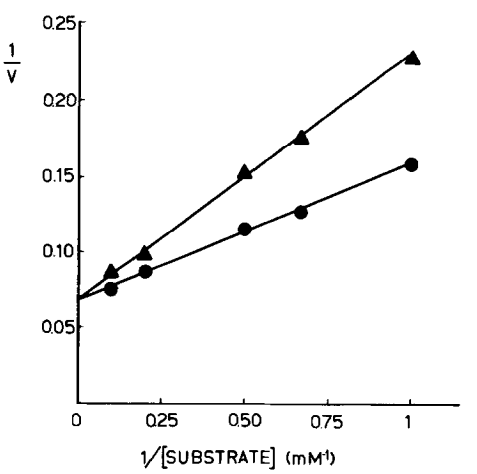
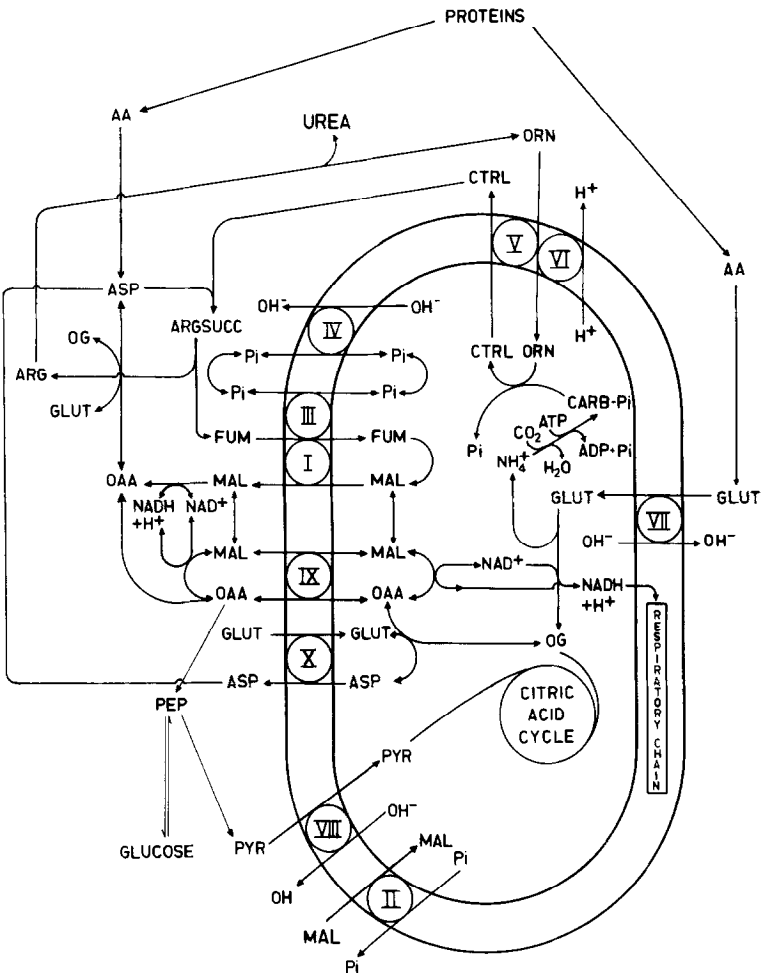



Fig. 4 The dependence of the rate of the malate/OAA and fumarate/OAA exchanges on the external substrate concentration using the double reciprocal plot.
Mitochondria (2 mg protein) were preincubated in 2.0 ml of standard medium and 0.2 mM NADH. After incubation for 1 min, 2 units of malate dehydrogenase were added followed 30 sec later by malate (▲) or fumarate (●) at the indicated concentrations. The rate V is expressed as nmoles NADH oxidized/min x mg mitochondrial protein.




Scheme 1 : Urea cycle and mitochondrial carriers.
I = fumarate/malate carrier; II = dicarboxylate carrier; III = fumarate/phosphate carrier; IV = phosphate carrier; V = citrulline/ornithine carrier; VI = ornithine carrier; VII = glutamate/OH carrier; VIII pyruvate/OH carrier; IX malate/oxaloacetate carrier; X = glutamate/aspartate carrier.

carrier to cause OAA efflux. Although apparently at odds with the above mentioned results, there is a possibiliy that OAA efflux induced by fumarate occurs by means of the cooperation of both FUM/MAL (I) and MAL/OAA (IX) translocators, as described in Scheme 1. To confirm this, tests were made that no OAA efflux occurs caused by fumarate addition to mitochondria loaded with both thiocyanate and sulphite, penetrant inhibitors of fumarase and malate dehydrogenase respectively, and with aspartate and oxoglutarate, whose uptake into RLM provides the substrate pair for the activity of mitochondrial aspartate aminotransferase and subsequent OAA formation. Triton was

added as a control to show the presence of OAA in the mitochondrial matrix (not reported).

## DISCUSSION

The ability of fumarate to enter isolated RLM is demonstrated by means of both isotopic and, in more detail, by optical measurements. Fumarate uptake is found to be a carrier mediated process. The existence of two specific fumarate translocators in RLM is shown according to criteria commonly used to state the occurrence of a carrier mediated process, i.e. saturation kinetic, sensitivity of this process to the impermeable inhibitors, and ability of the substrate to cause intramitochondrial anion efflux.

Results reported in this paper, along with others which show the lack of fumarate inhibition on dicarboxylate, tricarboxylate and oxodicarboxylate uptake (5,26) lead to the conclusion that fumarate enters mitochondria via two separate carriers of its own, namely fumarate/malate and fumarate/phosphate translocators. These are distinguished from each other in view of the different values of Vmax and their differing sensitivity to butylmalonate, NEM and methylfumarate summarized in Fig. 2.

The low Km value for fumarate compared to intracellular concentration (27) suggests that the fumarate translocators operate at a rate similar to the Vmax under physiological conditions in ureogenesis (Scheme 1). Thus, owing to the low activity of extramitochondrial fumarase (11), fumarate translocation into mitochondria seemes likely to occur to yield aspartate by means of the coupled activity of the mitochondrial fumarase and of the isoenzymes of malate dehydrogenase and aspartate aminotransferase. Accordingly, the activity of the fumarate/malate translocator is roughly similar to that calculated for the urea cycle under similar experimental conditions (6). It should be noted that the high sensitivity of the fumarate/malate carrier to butylmalonate shown here may also account for much of the inhibition of ureogenesis by butylmalonate, suggested to be due only to the inhibition of the dicarboxylate carrier (6).

At this point, the OAA efflux induced by fumarate merits some explanation in terms of its role in both gluconeogenesis and cytosolic amino acid catabolism in hepatic cells. When taken up by mitochondria, fumarate derived from aminoacid catabolism might cause

OAA efflux in cytosol where PEPcK is located. Phosphoenol-pyruvate formation could subsequently start gluconeogenesis or else pyruvate may be successively formed via pyruvate kinase, in which case pyruvate uptake and oxidation into RLM could occur. Accordingly, carbon skeleton of aminoacids may be used both in the gluconeogenesis pathway and, in what is likely to be a less important way as an energy source in liver.

Finally, this paper also shows the ability of RLM to allow malate/oxaloacetate shuttle at a significant rate. Considering that the transfer rate of cytosolic reducing equivalents into mitochondria may be calculated as 32 nmoles NADH oxidized/min x mg protein in rat liver at 37 °C (28), the malate/OAA shuttle appears to play a significant role in this process.

Acknowledgements : The kind assistance of S. Fusilli is gratefully acknowledged.

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