Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 1-10

OXALOACETATE PERMEATION IN RAT KIDNEY MITOCHONDRIA: PYRUVATE/OXALOACETATE AND MALATE/OXALOACETATE TRANSLOCATORS

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Received March 6, 1985

SUMMARY The mechanism of oxaloacetate efflux from rat kidney mitochondria has been investigated in view of its possible role both in gluconeogenesis and in trasferring cytosolic reducing equivalents into mitochondria.
Thus reconstruction of the malate/oxaloacetate shuttle made possible by the oxaloacetate carrier has been made. Moreover the existence of a separate translocator able to allow a bidirectional α-cyanocinnamate-insensitive pyruvate/oxaloacetate exchange has been ascertained. This carrier is specific of gluconeogenetic organs in particulary of kidney, where it shows a marked affinity for pyruvate (Km = 0.45 mM and Vmax = 38 nmoles oxaloacetate effluxed/min x mg mitochondrial protein at 20°C). Some features of both pyruvate/oxaloacetate and malate/oxaloacetate exchanges are also described.

The existence of an OAA translocation may play a major role in glucose metabolism, especially in gluconeogenesis, providing OAA outside mitochondria where P-enolpyruvate carboxykinase (E.C. 4.1.1.32) is predominantly found (1), as well as in the transport of glycolytic reducing equivalents in mitochondria via malate/OAA shuttle.

In the last decade OAA permeability has been reported in RLM and RHM (2-5) and recently the reconstruction of the malate/OAA shuttle in the brain has also been described (6). OAA translocation has been demonstrated also in RKM (7), however its role in the glucose metabolism has not yet been investigated in details. Reconstruction, using RKM, has been made here of the malate/OAA

Abbreviations: AA, aminoacids; AOA, aminooxyacetate; ARS, arsenite; DHAP, dihydroxyacetonephosphate; GLIC-3-Pi, glyceraldheyde-3-phosphate; α-GP, α-glycerol-3-phosphate; α-GPDH, α-glycerol-3-phosphate NAD^+ oxidoreductase; GPT, glutamic-pyruvic transaminase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LACT, lactate; LDH, lactate dehydrogenase; MAL, malate; MDH, malate dehydrogenase; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; PC, pyruvate carboxylase; PEP, P-enolpyruvate; PYR, pyruvate; PDH, pyruvate dehydrogenase; RBM, rat brain mitochondria; RHM, rat heart mitochondria; RKM, rat kidney mitochondria; RLM, rat liver mitochondria.

0006-291X/85 $1.50

shuttle. Moreover it has been found that there is an additional OAA transport mechanism, namely the pyruvate/OAA carrier, which seems to be active in renal gluconeogenesis.

MATERIALS AND METHODS All the reagents used were from SIGMA, except α-cyano-cinnamate which was a gift of Prof. G. Paradies. Mitochondrial substrates were used as Tris salts at pH 7.0-7.4. Rat kidney mitochondria were isolated according to (8) using a medium consisting of 0.25 M sucrose, 20 mM Tris-HCl pH 7.25, 1 mM EGTA. Isolated mitochondria showed relatively low respiratory control in agreement with (9). The mitochondrial protein was determined by the Waddel & Hill method (10).

Pyruvate/OAA, malate/OAA and OAA/pyruvate exchanges were monitored at 20°C by first placing mitochondria (about 1 mg protein) in each of two cuvettes suspended in 2.0 ml of standard medium containing 0.2 M sucrose, 10 mM KCl, 20 mM HEPES-Tris pH 7.20, 1 mM $MgCl_2$. The cuvette used as reference was put in a Beckman DU-7 HS spectrophotometer and its absorbance at 340 nm was subtracted from that of the other cuvette in which 0.2 mM NADH has been added. It was made sure that during each experiment the absorbance value of the reference cuvette, equalized to 0, was constant. Additions were made with rapid mixing and changes in the NADH absorbance automatically recorded in the display. Copy of the experimental trace was succesively obtained with every possible choice of the scale. Control experiments were made with Hitachi Perkin-Elmer 557 spectrophotometer to confirm the applicability of the procedure reported. The absorbance change rates were obtained as tangent at the initial part of the experimental curve and expressed as ΔA_{340}/min. ε_{340} value measured for NADH under our experimental conditions was found to be 6.2 $mM^{-1}cm^{-1}$.

RESULTS

In the phase outside mitochondria OAA concentration can be considered negligible, since no change of absorbance decrease occurs following MDH (E.C. 1.1.1.37) addition to RKM in the presence of 0.2 mM NADH which is slowly oxidized in the presence of the mitochondrial suspension (Fig. 1). Further addition of pyruvate (in the presence of ATP and HCO_3^-) (A) or malate (B) (1 mM each), which have no effect when added alone, produces a rapid decrease of absorbance, showing appearance of OAA outside the mitochondria.

A possible explanation for these results is the following (scheme 1, 2): a) pyruvate may enter mitochondria via its own carrier (for ref. see 11) in exchange with OH^- (12). Inside the matrix pyruvate is oxidized via PDH (E.C. 1.2.7.1) to acetylCoA. In the presence of ATP, OAA is subsequently formed by means of acetylCoA-activated pyruvate carboxylase (E.C. 6.4.1.1) and in turn it goes out of the mitochondria in a carrier mediated process in exchange with further pyruvate. b) Malate may enter mitochondria via dicarboxylate carrier (13) in exchange with catalitic amounts of endogenous phosphate. Once inside the mitochondria, malate is oxidized to OAA by intramitochondrial

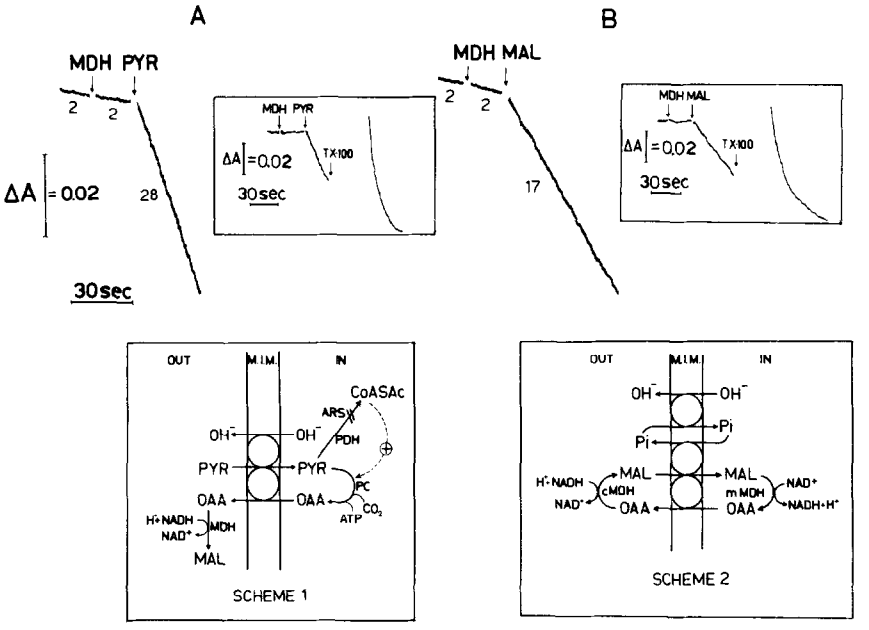

FIG. 1 **Appearance of oxaloacetate in the incubation medium induced by the addition of pyruvate or malate to rat kidney mitochondria.**
RKM (1 mg protein) were incubated at 20°C in 2.0 ml of standard medium in the presence of 0.2 mM NADH and 1 mM $NaHCO_3$ plus 1 mM ATP (in A) and 1 mM phosphate (in B). Where indicated, additions were as follows: 2 units of malate dehydrogenase from pig heart (MDH), 1 mM malate (MAL), 1 mM pyruvate (PYR), 0.5 % Triton X-100 (TX-100).
The trace shows the decrease in A_{340} and the figures alongside give the rate of NADH oxidation in nmoles/min x mg mitochondrial protein.
In the insets the traces of Triton experiments are shown separately as described in the Methods.

MDH. The reaction equilibrium is shifted toward malate oxidation by OAA efflux mediated by the OAA carrier in exchange with further malate (7).

The ability of pyruvate or OAA to cause malate efflux was also tested according to (7) in the same experiment. However, it was found that contrarily to OAA, in the presence of malic enzyme, pyruvate caused no reduction of externally added NADP, i.e. no malate efflux occurred (not shown).

The occurrence of the OAA efflux is *per se* a good indication in favour of the existence of carrier mediated processes. Thus use was made of Triton X-100 to investigate the rate limiting step of the measured absorbance decrease caused by both pyruvate/OAA and malate/OAA exchanges (see insets of Fig. 1). NADPH was used instead of NADH to avoid NADH oxidation by NADH dehydrogenase after the dissolution of mitochondria by Triton. About five times increase in NADPH oxidation rate was found in both cases after Triton

addition to RKM which were extruding OAA in the outside phase, suggesting that the rate of absorbance decrease reflects anion exchanges across the mitochondrial membrane.

Comparison has been made of mitochondria from heart, kidney, liver and brain regarding their ability to allow OAA efflux after pyruvate addition. At the same pyruvate concentration (1 mM) no efflux was found from RHM, whereas the relative rate of NADH oxidation were 26, 8, 2 nmoles NADH oxidized/min x mg protein for RKM, RLM and RBM, respectively.

To gain further insight into pyruvate/OAA and malate/OAA exchanges the effect of different compounds was investigated. Arsenite was found to inhibit OAA efflux induced by pyruvate, but no by malate. Moreover, no OAA efflux was found when pyruvate was added in the absence of ATP or HCO_3^-.

The effect of both α-cyanocinnamate and impermeable inhibitors of mitochondrial translocators such as phenylsuccinate, bathophenanthroline and mersalyl on either pyruvate/OAA or malate/OAA exchanges is reported in Fig. 2. α-cyano-

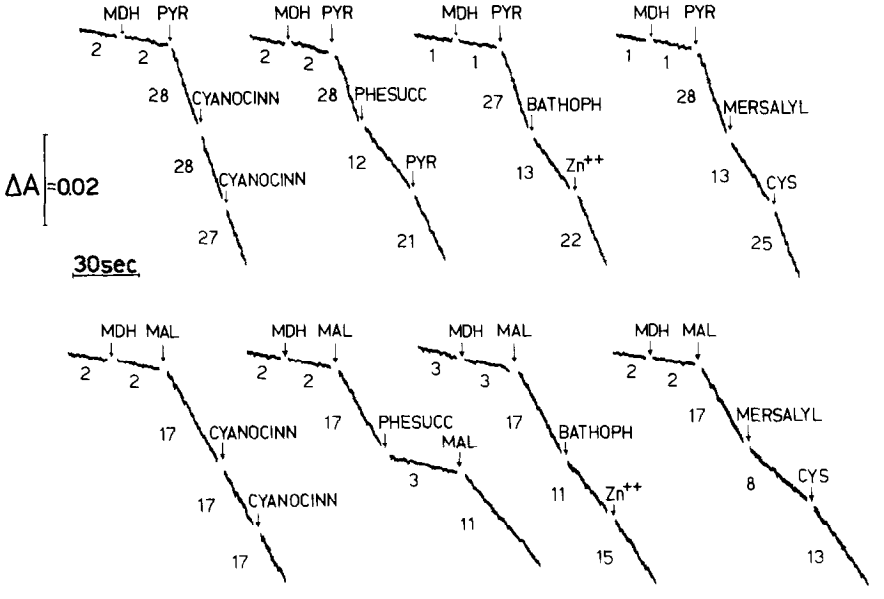

FIG. 2 Effect of α-cyanocinnamate, phenylsuccinate, bathophenanthroline and mersalyl on pyruvate/OAA and malate/OAA exchanges.
RKM (1 mg protein) were incubated at 20°C in 2.0 ml of standard medium in the presence of 0.2 mM NADH and 1 mM $NaHCO_3$ plus 1 mM ATP (in A) and 1 mM phosphate (in B). Where indicated, additions were as follows: 2 units of malate dehydrogenase (MDH), 1 mM malate (MAL), 1 mM pyruvate (PYR), 0.5 mM α-cyanocinnamate (CYANOCINN), 20 mM phenylsuccinate (PHESUCC), 30 μM bathophenanthroline (BATHOPH), 20 μM $ZnCl_2$ (Zn^{++}), 1 mM mersalyl and 1 mM cysteine (CYS). The trace shows the decrease in A_{340} and the figures alongside give the rate of NADH oxidation in nmoles/min x mg mitochondrial protein.

cinnamate, a powerful inhibitor of pyruvate transport (for ref. see 11), inhibits neither exchanges. It should be noted that under the same experimental conditions pyruvate oxidation, which was measured polarographycally, resulted completely blocked. Phenylsuccinate inhibits both exchanges; 60 and 95 % inhibition is found for pyruvate and malate (1 mM each), respectively. These inhibitions are partially removed by 10 mM substrates according to a competitive nature of inhibition. Both the metal-complexing agent bathophenanthroline (14,15) and the thiol reagent mersalyl (16) inhibit OAA efflux; the inhibition is partially reversed by Zn^{++} ions or cysteine (20 uM and 1 mM, respectively) which per se had no effect on the control (not shown). The inhibition by impermeable compounds confirms the existence of carrier-mediated antiport.

A test was carried out to ascertain whether pyruvate efflux from mitochondria could be caused by OAA or malate addition (Fig. 3). To do this mitochondrial pyruvate concentration was increased by incubating RKM with alanine and 2-oxo-

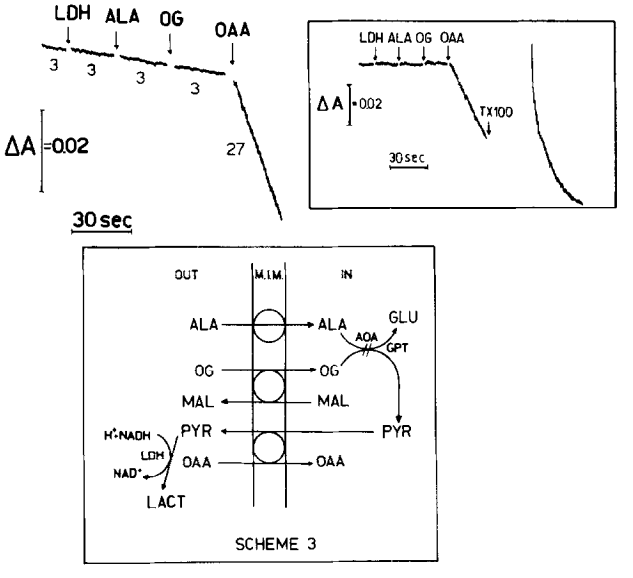

FIG. 3 Appearance of pyruvate in the incubation medium induced by the addition of oxaloacetate to rat kidney mitochondria.

RKM (1 mg protein) were incubated at 20°C in 2.0 ml of standard medium in the presence of 0.2 mM NADH, 1 mM sodium arsenite and 0.1 mM α-cyanocinnamate. Where indicated, additions were as follows: 1 unit of lactate dehydrogenase from hog muscle (LDH), 1 mM alanine (ALA), 0.5 mM 2-oxoglutarate (OG), 0.1 mM oxaloacetate, 0.5 % Triton X-100 (TX-100).

The trace shows the decrease in A_{340} and the figures alongside give the rate of NADH oxidation in nmoles/min x mg mitochondrial protein.

In the inset the traces of Triton experiment are shown separately as described in the Methods.

glutarate whose uptake into mitochondria (17,18) provides the substrate pair for intramitochondrial glutamate-pyruvate transaminase (E.C. 2.6.1.2). Arsenite and α-cyanocinnamate were also present to inhibit PDH and pyruvate transport via its own carrier, respectively. Pyruvate efflux occurs following OAA addition to RKM as revealed by the decrease of NADH absorbance in the presence of LDH (E.C. 1.1.1.27) (scheme 3). It should be noted that the addition of both alanine and 2-oxoglutarate to RKM incubated with LDH caused per se no change in NADH absorbance, moreover in the presence of amino-oxyacetate, transaminase inhibitor able to enter mitochondria (19), the change of absorbance was largely inhibited. In the same experiment it was verified that no change of absorbance was due to possible leakage of intramitochondrial MDH or to OAA spontaneous decarboxylation. Malate failed to cause appreciable pyruvate efflux (not shown). The Triton experiment shows (see inset of Fig. 3) that the substrate exchange across mitochondrial membrane is also in this case the rate limiting step of the measured NADPH oxidation.

The dependence of the NADH oxidation rate on the externally added pyruvate, malate or OAA was investigated in the same experiment in the presence of MDH (in the case of both pyruvate and malate) (A) or of LDH in the case of OAA (B). Saturation kinetics were found with a Km value, i.e. the substrate concentration which gives half maximum NADH oxidation rate, equal to 0.45 mM, 0.28 mM and 0.08 mM in the case of pyruvate/OAA, malate/OAA and OAA/pyruvate exchanges, respectively. Vmax values were 38, 20 and 38 nmoles NADH oxidized/min x mg mitochondrial protein (Figure 4).

In order to gain further insight into pyruvate/OAA and malate/OAA translocators, the nature of the inhibition by bathophenanthroline and by mersalyl was also determined by means of double reciprocal plots. The addition of bathophenanthroline (15 μM and 30 μM in A and B, respectively) did not change the Vmax value, but increased the Km values in both cases (Ki values were 6 μM and 12 μM for pyruvate/OAA and malate/OAA, respectively), according to a competitive inhibition. On the other hand mersalyl (0.25 mM) appears to be a non-competitive inhibitor as shown by the decrease of Vmax and by the lack of change in Km values (Ki values were 2.2 mM and 0.8 mM in the case of pyruvate/OAA and malate/OAA, respectively) (Figure 5).

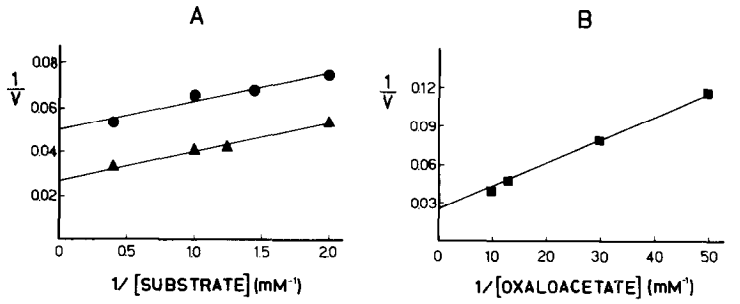

FIG. 4 The dependence of the rate of the pyruvate/OAA, malate/OAA and OAA/pyruvate exchanges on the external substrate concentration using the double reciprocal plot.
Pyruvate (▲), malate (●) (A) or oxaloacetate (■) (B) were added at the indicated concentrations. The experiment was carried out as described in Fig. 1. The rate V is expressed as nmoles NADH oxidized/min x mg mitochondrial protein Mitochondrial protein was 1 mg.

Both phenylsuccinate and phthalonate (7) resulted in a competitive inhibition (not shown).

DISCUSSION

This paper shows that OAA efflux from RKM can occur by means of two separate mechanisms namely malate/OAA and pyruvate/OAA antiports. The malate/OAA exchange can be mediated by the OAA carrier (7) whereas a translocator which had not been previously determined causes OAA efflux from mitochondria in ex-

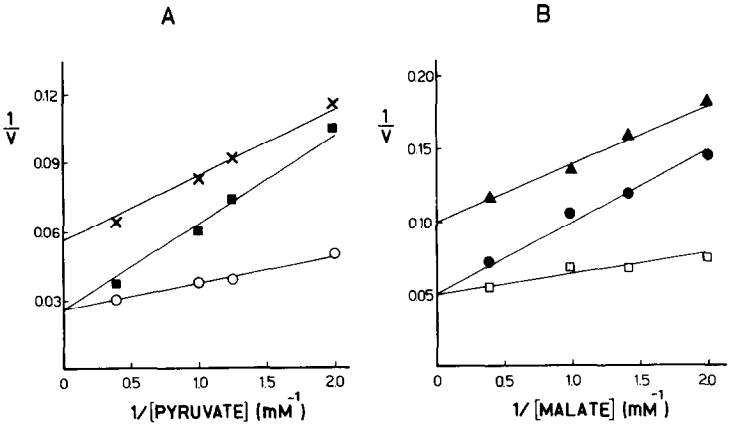

FIG. 5 Kinetics analysis of the inhibition of pyruvate/OAA and malate/OAA exchanges by bathophenanthroline or mersalyl using the double reciprocal plot.
RKM (1.5 mg protein) were incubated at 20°C in 2.0 ml of standard medium. The experiment was carried out as described in Fig. 1.
In A and B, pyruvate and malate were added at the indicated concentrations in the absence (○-□) or in presence of bathophenanthroline (■-●) 15 and 30 μM in A and B, respectively, and mersalyl 0.25 mM (x-▲).
The rate V is expressed as nmoles NADH oxidized/min x mg mitochondrial protein.

change with pyruvate. This carrier is found to be different from the pyruvate carrier as shown by the lack of inhibition by α-cyanocinnamate. The occurrence of carrier mediated transport was demonstrated according to criteria commonly used, i.e. saturation kinetics, specificity of the exchanges and inhibition by impermeable compounds. The above carriers can be distinguished owing to the different values of Vmax and more specifically in view of the fact that malate cannot cause a pyruvate efflux from the mitochondria and vice versa. Pyruvate/OAA antiport was shown to be the rate limiting step of NADH oxidation rate. Consistently the rate of malate/OAA exchange also is shown here to depend on the malate/OAA antiport, contrarily to that previously found for OAA/malate antiport (7). Interestingly pyruvate/OAA antiport appears to be bidirectional in vitro, however the possible physiological occurrence of pyruvate efflux from RKM caused by OAA would require more detailed investigation. It should be noted that under the experimental conditions of Fig. 3 OAA/2-oxoglutarate exchange may also occur. Both the reported translocators are inhibited in the same way, even if in different degree, by bathophenanthroline and mersalyl. This inhibition may be due to their ability to react with metal ions or -SH groups as shown by the reversal obtained in the presence of Zn^{++} or cysteine, respectively. The competitive nature of the inhibition by bathophenanthroline suggests that a metal ion/s is located at or near the substrate binding sites, whereas thiol/s are proposed to be located far from the substrate binding sites according to the non-competitive inhibition found.

Both pyruvate/OAA and malate/OAA translocators are inhibited competitively by the dicarboxylate analogues such as phenylsuccinate and phthalonate; this finding is in favour of a single site carrier which is suggested to bind both dicarboxylates and oxoacids.

The physiological role played by malate/OAA and pyruvate/OAA translocators in RKM is related to the mechanism by which shuttling systems carry out the oxidation of NADH generated in the cytoplasm and by which OAA efflux from mitochondria of gluconeogenetic organs occurs.

This paper, in fact, shows that malate/OAA shuttle can occur in vitro also in kidney. Thus occurrence of malate/OAA shuttle in addition to the reported malate/aspartate (19,20) and α-glycerol-Pi/dihydroxyacetone-Pi (21) may be possible. The activity of this shuttle is similar to that reported for

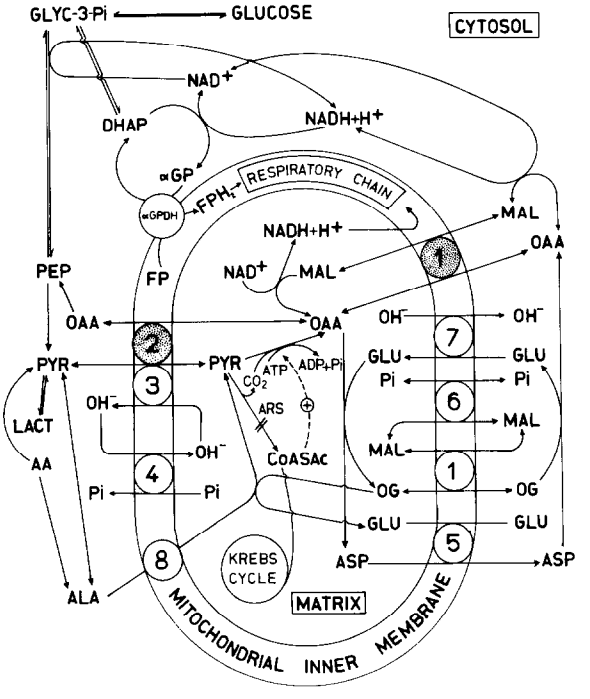

FIG. 6 1 = oxodicarboxylate carrier; 2 = pyruvate/oxaloacetate carrier; 3 = pyruvate carrier; 4 = phosphate carrier; 5 = glutamate/aspartate carrier; 6 = dicarboxylate carrier; 7 = glutamate/OH^- carrier; 8 = alanine carrier.

α-glycerol-Pi/dihydroxyacetone-Pi shuttle (Vmax values are 20 and 23 nmoles NADH oxidized/min x mg protein) under roughly similar experimental conditions.

The activity of pyruvate/OAA translocator can account for OAA efflux from mitochondria in gluconeogenesis. Consistently pyruvate/OAA exchange was found to occur both in RLM and more significantly in RKM, whereas no or very low activity was found in RHM or RBM, respectively. Inspite of the fact that only 15 % of renal gluconeogenesis seems to be insensitive to α-cyano-OH-cinnamate (22), the high activity of this translocator, which appears to regulate the rate of OAA efflux in exchange with pyruvate (see Triton experiment) could suggest a major role of this process in gluconeogenesis. In fact possible inhibition by α-cyanocinnamate on reaction other than substrate transport cannot be totally excluded.

The overall picture of mitochondrial permeation to anionic substrates in kidney derived from this and related papers is shown in figure 6.

ACKNOWLEDGMENTS: The helpful cooperation of dr. C. Mancini who participated at the first stages of this work and the skilful technical assistance of Mr. P. Petragallo are gratefully acknowledged.
This research was partially supported by 84/2929 grant of Italian M.P.I.

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