D-Lactate transport and metabolism in rat liver mitochondria

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In the present study we investigated whether isolated rat liver mitochondria can take up and metabolize D-lactate. We found the following: (1) externally added D-lactate causes oxygen uptake by mitochondria [P/O ratio (the ratio of mol of ATP synthesized to mol of oxygen atoms reduced to water during oxidative phosphorylation) = 2] and membrane potential ($\Delta\psi$) generation in processes that are rotenone-insensitive, but inhibited by antimycin A and cyanide, and proton release from coupled mitochondria inhibited by α-cyanocinnamate, but not by phenylsuccinate; (2) the activity of the putative flavoprotein (D-lactate dehydrogenase) was detected in inside-out submitochondrial particles, but not in mitochondria and mitoplasts, as it is localized in the matrix phase of the mitochondrial inner membrane; (3) three novel separate translocators exist to mediate D-lactate traffic across the mitochondrial inner membrane: the D-lactate/H⁺ symporter, which was investigated by measuring fluorimetrically the rate of endogenous flavin reduction, the D-lactate/oxoacid antiporter (which mediates both the D-lactate/pyruvate and D-lactate/oxaloacetate exchanges) and D-lactate/malate antiporter studied by monitoring photometrically the appearance of the D-lactate counteranions outside mitochondria. The D-lactate translocators, in the light of their different inhibition profiles separate from the monocarboxylate carrier, were found to differ from each other in the $V_{\max}$ values and in the inhibition and pH profiles and were shown to regulate mitochondrial D-lactate metabolism *in vitro*. The D-lactate translocators and the D-lactate dehydrogenase could account for the removal of the toxic methylglyoxal from cytosol, as well as for D-lactate-dependent gluconeogenesis.

Key words: antiporter, dehydrogenase, flavoprotein, gluconeogenesis, methylglyoxal pathway, symporter.

INTRODUCTION

Despite the huge number of papers dealing with the mitochondrial bioenergetics and transport, knowledge of the role of mitochondria in energy metabolism is far from being completely elucidated. This certainly applies to lactate isomer metabolism [1]. In particular, although L-lactate oxidation via mitochondrial L-lactate dehydrogenase (L-LDH) [2–4] has been shown in mammalian mitochondria from different sources and the occurrence of the L-lactate/pyruvate shuttle has been recently reported in rat heart [5], the role of mitochondria in D-lactate metabolism still remains obscure. D-Lactate is formed in the liver cytosol in the methylglyoxal pathway [6]; moreover, it can be derived from other tissues via the bloodstream by means of carrier-mediated transport [7–11]. D-Lactate levels in rat liver are consistently about one-sixth of those of L-lactate under physiological conditions [12]. On the other hand, the concentration of rat D-lactate in plasma was found to increase after running exertion [13] and in diabetes and starvation [12], but to decrease in aging [14]. Oxidation to CO_2 as well as gluconeogenesis from D-lactate have been already reported [15,16], but how it occurs has been not yet investigated in detail. Rat liver mitochondria (RLM) can produce D-lactate from lactoylglutathione owing to the existence of glyoxalase II in the matrix [17–20], perhaps requiring the existence of mitochondrial D-LDH (D-LDH) activity and/or D-lactate transport outside mitochondria. On the other hand, cytosolic D-lactate could be transported and metabolized in mitochondria. In this respect, although carrier-mediated transport in mammalian mitochondria has been shown for many metabolites [21–25] and organic compounds [26], mitochondrial permeability to D-lactate is unknown.

Thus in the present study we investigated whether, and how, D-lactate metabolism occurs in RLM. We show that RLM possess three separate translocators, namely the D-lactate/H⁺ symporter, the D-lactate/oxoacid antiporter (which mediates both the D-lactate ⇌ pyruvate and D-lactate ⇌ oxaloacetate exchanges) and the D-lactate/malate antiporter, which can transport D-lactate from the cytosol to the matrix where D-lactate is oxidized to pyruvate by the putative D-LDH (D-LDH; EC 1.1.1.28) located in the inner face of the mitochondrial inner membrane. As a result of D-lactate-dependent metabolism and traffic across the mitochondrial membrane, both oxaloacetate and malate, besides pyruvate, are available in the extramitochondrial phase, thus making D-lactate a gluconeogenic substrate.

EXPERIMENTAL

Materials

Rabbit muscle L-LDH (L-LDH; EC 1.1.1.27), chicken liver 'malic' enzyme (EC 1.1.1.40), NADH, NADP⁺, rotenone, antimycin A, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), valinomycin, nigericin, KCl, mannitol, phenazine methosulphate (PMS), dichloroindophenol (DCIP), *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), ADP, D-lactic acid,

L-lactic acid, succinic acid, malic acid, glutamic acid, *N*-methylglutamic acid, DL-*β*-amino-*N*-butyric acid, *N*-methylaspartic acid, *α*-cyano-4-hydroxycinnamic acid (*α*CCN⁻) and ascorbic acid, D-lactic acid methyl ester, bathophenanthroline (BF) and *N*-methyL-DL-alanine were obtained from Sigma; methylmaleic acid and phenylsuccinic acid were obtained from Fluka. Phthalonic acid was prepared as described in [27]; pig heart malate dehydrogenase (EC 1.1.1.37), sucrose, Triton X-100, Hepes, Tris and sodium arsenite were from Baker; digitonin was from Calbiochem.

All chemicals were of purest grade available and were used as Tris salts at pH 7.0–7.4 adjusted with either Tris or HCl. Valinomycin, nigericin, rotenone, antimycin A and FCCP were dissolved in ethanol.

Isolation of RLM

RLM were isolated from male Wistar rats (150–200 g body weight) as described in [28], using a medium consisting of 0.25 M sucrose and 20 mM Tris/HCl, pH 7.25. The final mitochondrial pellet was suspended in the same medium to obtain 60–70 mg of protein/ml. The mitochondrial protein was measured as described in [29].

Isolation of mitoplasts

Mitoplasts were isolated as described in [26], using RLM isolated in a medium containing 70 mM sucrose, 220 mM mannitol, 2 mM Hepes, pH 7.25, and 0.05 % BSA (medium A). The final mitochondrial pellet (about 250 mg of protein) was suspended in 5 ml of medium A to which 5 ml of a solution of digitonin (6 mg/ml in medium A) was added, the mixture being shaken at 0 °C for 15 min. A 30 ml portion of medium B, consisting of 1 litre of medium A plus 500 mg of BSA, was added, and mitoplasts collected by centrifugation (10 000 *g* for 10 min), then subsequently suspended in medium B (20 ml), centrifuged again under the same conditions, and finally resuspended in medium B (maximum volume 1 ml). Supernatants of the first and the second centrifugations were centrifuged (105 000 *g* for 60 min, 4 °C) to obtain intermembrane-space and mitochondrial-outer-membrane fractions.

The absence of intact mitochondria in the sample was checked by assaying adenylate kinase (EC 2.7.4.3), a marker enzyme of the intermembrane space, with mitoplast integrity confirmed by verifying the lack of glutamate dehydrogenase (EC 1.4.1.3) activity in suspension.

Preparation of EDTA submitochondrial particles

EDTA submitochondrial particles (ESMP) were prepared as described in [30]. RLM, frozen for 3 days, were suspended (10 mg/ml) in a medium consisting of 0.25 M sucrose, 4 mM potassium EDTA, pH 8.4–8.8. Mitochondrial suspension (about 60 ml) was sonicated with the microtip probe of a Branson sonifier at 70 W until it became translucent. The suspension was subsequently centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant obtained was collected and centrifuged at 105 000 *g* for 30 min at 4 °C. The supernatant was washed with 0.25 M sucrose and then centrifuged again at 105 000 *g* for 10 min at 4 °C. The submitochondrial particles obtained proved to be 90–95 % inside-out, as shown by assaying NADH oxidation in either the absence or presence of 0.1 % Triton X-100.

Cytosolic fraction preparation

The cytosolic fraction was obtained by centrifuging (105 000 *g* for 60 min at 4 °C) the supernatant obtained in the RLM

isolation. L-LDH and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were assayed as described in [31] and [32] respectively.

Safranine response assay

Safranine O response was monitored as described in [24,33]. Measurements were carried out at 25 °C in 2.0 ml of RLM isolation medium containing 9.6 μM safranine O and 1 mg of mitochondrial protein.

Oxygen-uptake studies

Oxygen-uptake measurements were carried out at 25 °C in 1.5 ml of a medium consisting of 210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 20 mM Tris/HCl, pH 7.4, 3 mM MgCl$_2$, 5 mM KH$_2$PO/K$_2$HPO$_4$ by means of a Gilson 5/6 oxygraph using a Clark electrode.

D-LDH assay

The D-LDH assay was performed photometrically by means of a PerkinElmer Lambda-5 spectrophotometer at 600 nm, as described in [34], at 25 °C. Briefly the mitochondrial sample was incubated for 2 min in 2 ml of a standard medium consisting of 0.2 mM sucrose, 10 mM KCl, 20 mM Hepes/Tris, pH 7.2, 1 mM MgCl$_2$ in the presence of 30 μM PMS and 50 μM DCIP. D-LDH activity was assayed by measuring the decrease in A_{600} due to DCIP reduction that occurs when 15 mM D-lactate was added. The activity was expressed as nmol of DCIP reduced/min per mg ($\epsilon_{600} = 21$ mM$^{-1} \cdot$ cm^{-1}).

Swelling experiments

Mitochondrial swelling was monitored at 25 °C at 546 nm by means of a PerkinElmer Lambda-5 spectrophotometer. Mitochondria (1 mg of protein) were rapidly added to isotonic solutions of ammonia salts the pH values of which were adjusted to 7.2, and the decrease in the absorbance continuously recorded.

Proton-movement measurements

Proton movement across the mitochondrial membrane was monitored, as previously reported [24,33], by means of a Gilson 5/6 Oxygraph equipped with a Gilson pH 5 Servo Channel electrode which allows for continuous monitoring and direct recording of mitochondrial-suspension pH changes during the assays. The calibration was made by using HCl.

Mitochondria were added, at 25 °C, to 1.5 ml of the proton medium consisting of 100 mM NaCl, 10 mM MgCl$_2$, 1 mM EGTA/Tris and 2 mM Tris/HCl, pH 7.0.

Fluorimetric and photometric assay

Changes in the redox state of either flavin or mitochondrial nicotinamide nucleotide were monitored fluorimetrically using a PerkinElmer LS-5 luminometer. Excitation and emission wavelengths of 450–520 nm and 334–456 nm respectively were used. The NAD(P)H fluorescence was calibrated as described in [22–24].

D-Lactate uptake was monitored by measuring the intramitochondrial FAD/FMN reduction caused by externally adding the substrate to RLM (1 mg). The RLM had previously been incubated in 2 ml of standard medium in the presence of 1.25 μM FCCP to oxidize intramitochondrial flavins and, 2 min later, 2 μg of rotenone, 1.5 μg of antimycin A and 1 mM cyanide were added to prevent the oxidation of the newly synthesized FADH$_2$/FMNH$_2$ via the respiratory chain.

Pyruvate uptake was monitored by measuring the reduction of intramitochondrial $NAD(P)^+$ caused by externally adding the substrate to mitochondria previously incubated with 1.25 μM FCCP and added 3–5 min later with 2 μg of rotenone to prevent the oxidation of the newly synthesized NAD(P)H via the respiratory chain.

Pyruvate, malate and oxaloacetate (OAA) appearance outside mitochondria was monitored by using the pyruvate-detecting system (PDS; consisting of 200 μM NADH plus 1 unit of L-LDH), the oxaloacetate-detecting system (ODS; consisting of 200 μM NADH plus 1 unit of MDH) and the malate-detecting system (MDS; consisting of 200 μM $NADP^+$ plus 0.2 unit of 'malic' enzyme) and then monitoring photometrically NADH oxidation (in the case of either pyruvate or oxaloacetate) and $NADP^+$ reduction (in the case of malate appearance), caused by externally added D-lactate (or OAA), which, *per se*, proved to have no effect on the enzymic reactions and on the absorbance measured at 334 nm. In this case the ϵ_{334} value measured for both NADH and NADPH under our experimental conditions was found to be 6.5 $mM^{-1} \cdot cm^{-1}$.

Controls were set up to ensure that none of the compounds used in the present study affected the enzymes used to reveal the appearance of metabolites outside the mitochondria.

In each experiment, the intactness of the mitochondrial inner membrane was checked by measuring the rate of NADH oxidation in the absence of any substrate. When the rate was more than 5% of that measured following metabolite efflux, mitochondria were not used. In each experiment NADH was added to both the sample and the reference cuvette, and the rate of NADH oxidation was measured as the difference between the same rate measured in the sample cuvette and in the reference cuvette, thus permitting one to obtain the correct rate of the absorbance change due to the reactions outside the mitochondria (see [25]).

The rates of both fluorescence and absorbance change were obtained as tangents to the initial parts of the progress curves and expressed as arbitrary units of FAD/FMN reduced/mg of mitochondrial protein and as nmol of NADH oxidized ($NADP^+$ reduced)/mg of mitochondrial protein respectively.

RESULTS

D-Lactate oxidation in RLM

We investigated whether isolated RLM can oxidize D-lactate. Mitochondria were added together with D-lactate (5 mM), and measurements of oxygen uptake made under both State 4 and State 3 conditions in the presence of 0.1 mM ADP; oxygen uptake was found with a respiratory control index (State 3 rate/State 4 rate ratio) of 5 and P/O ratio (the ratio of mol of ATP synthesized to mol of oxygen atoms reduced to water during oxidative phosphorylation) of 2.1 (Figure 1A). In ten different experiments, P/O ratios were calculated for both D-lactate and for succinate (used as a control): they were 2.05 ± 0.2 and 1.98 ± 0.1, i.e. not statistically different as judged by Student's t test. The Complex I inhibitor rotenone completely blocked oxygen uptake caused both by the substrate pair glutamate + malate (each 5 mM) and by L-lactate (results not shown), but permitted D-lactate (5 mM) oxidation in State 4; this was completely inhibited by antimycin A, a powerful inhibitor of Complex III. Oxygen consumption was restored by adding ascorbate (5 mM) plus TMPD (0.5 mM), but completely inhibited by cyanide (1 mM) (Figure 1B). In order to ascertain whether D-lactate oxidation involves the monocarboxylate carrier, pyruvate and D-lactate oxidation in State 4 were compared in the same experiment. Pyruvate oxidation was found to
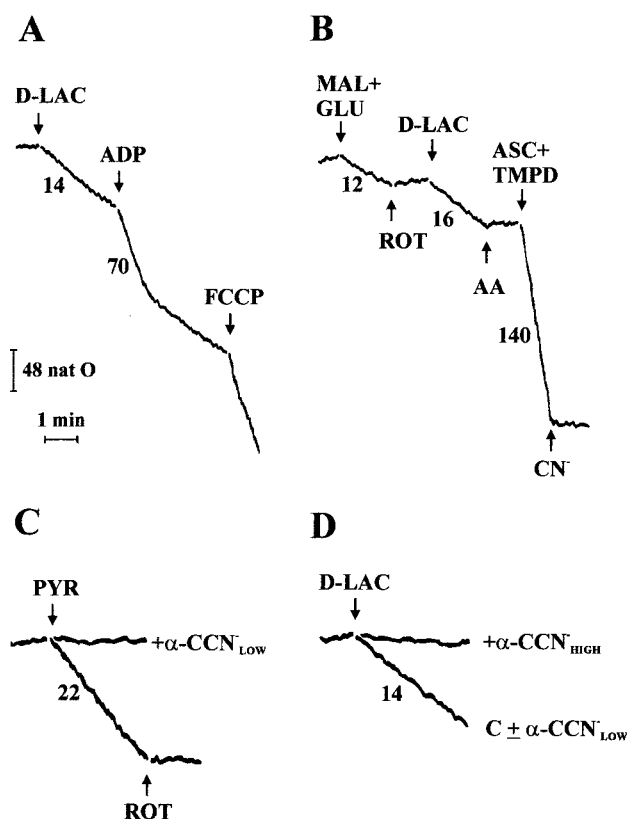

Figure 1 Oxygen uptake by RLM added with either D-lactate or pyruvate

RLM (1 mg of protein) were suspended at 25 °C in 1.5 ml of a medium consisting of 210 mM mannitol, 70 mM sucrose, 3 mM $MgCl_2$, 0.1 mM EDTA, 5 mM KH_2PO_4/K_2HPO_4 and 20 mM Tris/HCl, pH 7.4, and the amount of oxygen was measured as a function of time. At the arrows the following additions were made: D-lactate (D-LAC, 5 mM) (**A**), malate plus glutamate (MAL + GLU, each 5 mM), rotenone (ROT, 2 μg), antimycin A (AA, 1.5 μg), ascorbate (ASC, 5 mM) plus TMPD (0.5 mM), KCN (CN^-, 1 mM) (**B**) and pyruvate (PYR, 0.5 mM). In (**C**) and (**D**), either 25 μM or 1 mM αCCN^- (α-CCN^-_{LOW} or α-CCN^-_{HIGH} respectively), when present, was added 1 min before the substrate addition. Numbers along the curves are rates of oxygen uptake expressed as n-atoms of O/min per mg of mitochondrial protein.

be completely prevented by an externally added low αCCN^- concentration (25 μM) and blocked by rotenone (Figure 1C), whereas oxygen uptake due to D-lactate was found to be essentially insensitive to 25 μM αCCN^-, but completely inhibited by a high αCCN^- concentration (1 mM) (Figure 1D).

The ability of D-lactate to generate membrane potential ($\Delta\psi$) as well as proton ejection from the mitochondrial matrix was checked by using safranine as $\Delta\psi$ probe and by directly measuring proton ejection outside mitochondria. $\Delta\psi$ generation was found as a result of the addition of D-lactate (5 mM) to RLM, as monitored by a decrease of safranine absorbance (Figure 2A). $\Delta\psi$ generation was prevented by antimycin A and abolished by the uncoupler FCCP. In the same experiment, either succinate (5 mM) or pyruvate (0.5 mM), which generated a $\Delta\psi$ with a higher rate with respect to D-lactate in a manner inhibited by antimycin and by rotenone respectively, were also added.

Proton movement across the mitochondrial membrane due to D-lactate addition was measured as a function of time. Proton release from mitochondria, due to D-lactate, succinate (5 mM each) and pyruvate (0.5 mM) addition to RLM, was observed up to 5 min. As expected, proton uptake by RLM due to FCCP addition was found (Figure 2B).
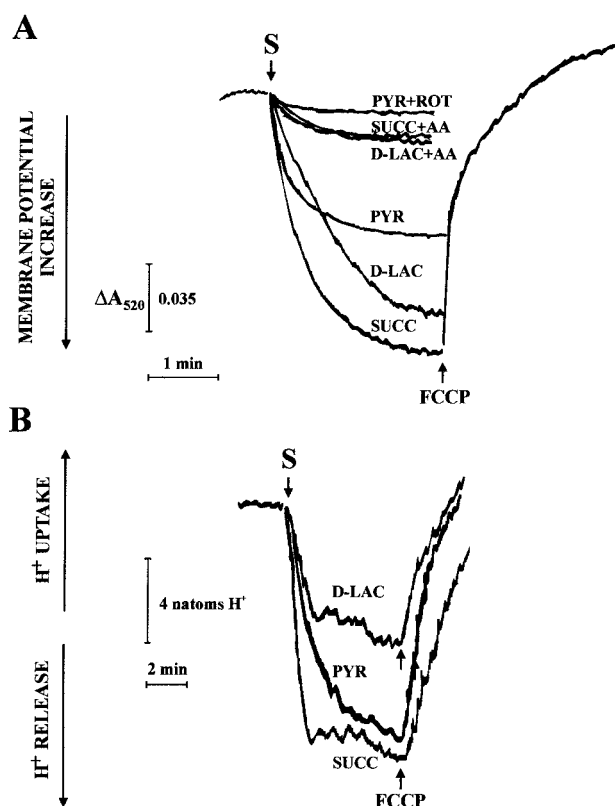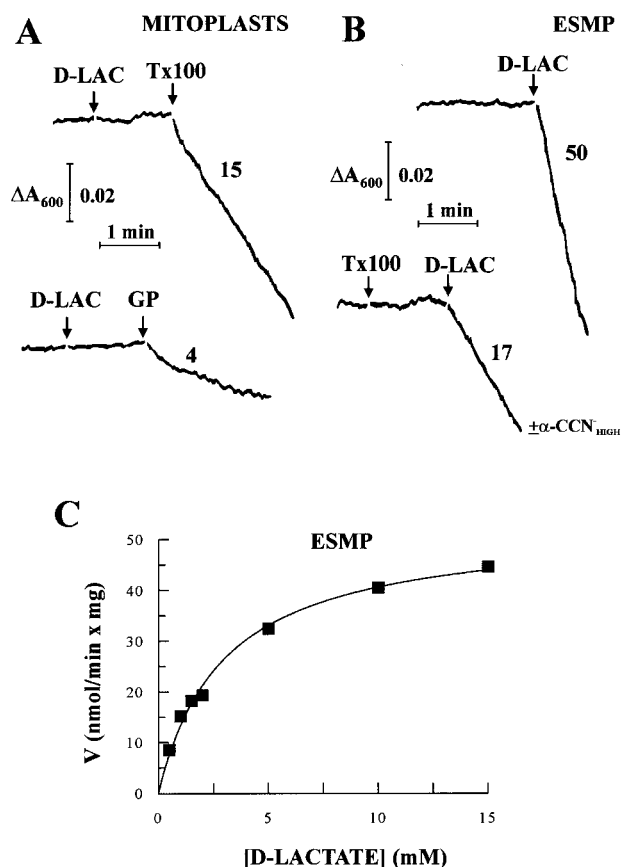
Figure 2 Changes in the mitochondrial Δψ measured as safranine response and proton movements accompanying D-lactate and pyruvate uptake into RLM

(**A**) RLM (1 mg of protein) were suspended at 25 °C in 2 ml of RLM isolation medium containing safranine (9.6 μM) in the presence of 2 μg of rotenone (in the case of succinate). The following additions were made: substrate (S), i.e. D-lactate (D-LAC, 5 mM), succinate (SUCC, 5 mM) or pyruvate (PYR, 0.5 mM), and FCCP (1.25 μM). Where indicated, rotenone (ROT, 2 μg) or antimycin A (AA, 1.5 μg) was added 2 min before PYR or D-LAC/SUCC respectively. Safranine response was monitored as described in the Experimental section. (**B**) RLM (1 mg of protein) were suspended at 25 °C in 1.5 ml of proton medium consisting of 100 mM NaCl, 10 mM MgCl₂, 1 mM EGTA/Tris, 5 mM Tris/HCl, pH 7.00, in the presence of 2 μg of rotenone (in the case of succinate). At the arrows the substrate (S), i.e. D-lactate (D-LAC, 5 mM), succinate (SUCC, 5 mM) or pyruvate (PYR, 0.5 mM) and FCCP (1.25 μM) were added. Proton movement was monitored as described in the Experimental section.

Figure 3 D-LDH activity assay in mitoplasts and submitochondrial particles (ESMP) and the dependence of the rate of D-lactate oxidation by ESMP on increasing D-lactate concentrations

(**A**) Mitoplasts (0.2 mg of protein) and (**B**, **C**) ESMP (0.05 mg of protein) were suspended at 25 °C in 2 ml of standard medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM MgCl₂, 20 mM Hepes/Tris, pH 7.2, in the presence of 30 μM PMS and 50 μM DCIP. D-LDH activity was monitored at 600 nm as described in the Experimental section. (**A**, **B**) At the arrows, D-lactate (D-LAC, 15 mM), glycerol 3-phosphate (GP, 2 mM) and Tx100 (0.5%) were added. In (**B**), α-CCN$_{HIGH}^-$, 1 mM, when present, was added 1 min before the D-lactate addition. Numbers along the curves are rates of D-lactate oxidation expressed as nmol of DCIP reduced/min per mg of protein. (**C**) D-Lactate was added at the indicated concentrations to ESMP with the rate (v) of DCIP reduction calculated as the tangent to the initial part of the progress curve and expressed as nmol/min per mg of ESMP protein.

In order to ascertain the localization of the enzyme that causes D-lactate oxidation, RLM, mitoplasts and inside-out ESMP were assayed for the occurrence of D-LDH activity as reported in the Experimental section. No decrease in DCIP absorbance was found when D-lactate (15 mM) was added to either RLM (results not shown) or mitoplasts (Figure 3A), this indicating the absence of D-LDH activity in the outer membrane, in the intermembrane space and in the outer side of the mitochondrial inner membrane. This was confirmed by direct assays carried out with the isolated fractions (results not shown). In a control experiment, 2 mM glycerol 3-phosphate was added to mitoplasts, resulting in an absorbance decrease with a rate of about 4 nmol/min per mg due to the activity of the glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) located in the outer side of the mitochondrial inner membrane.

Following the addition of Triton X-100 (Tx100; 0.5%), which dissolves the mitochondrial membranes, D-LDH activity was measured and found to be about 15–17 nmol/min per mg both in RLM and in mitoplasts. Interestingly, the D-LDH reaction

was noted as occurring at a rate of about 50 nmol/min per mg of protein after 15 mM D-lactate addition to ESMP (Figure 3B). Since these particles are inside-out [35], this shows that the protein that oxidizes D-lactate is associated with the inner side of the mitochondrial inner membrane. When Tx100 was added to ESMP, a strong decrease in the D-LDH reaction rate was observed ($v = 17$ nmol/min per mg of protein), suggesting a functional association of the putative D-LDH enzyme with the inner membrane. In the same experiment, a roughly similar decrease in succinate dehydrogenase activity was found after the addition of Tx100 to ESMP, whereas no significant change of absorbance was found owing to the addition of glycerol phosphate (results not shown). The localization of the putative D-LDH was consistent with the digitonin titration (results not shown): D-LDH activity was assayed in RLM as a function of the digitonin/protein ratio, essentially as described in [36]. As a control, the release of monoamine oxidase, adenylate kinase, succinate dehydrogenase and glutamate dehydrogenase, used as
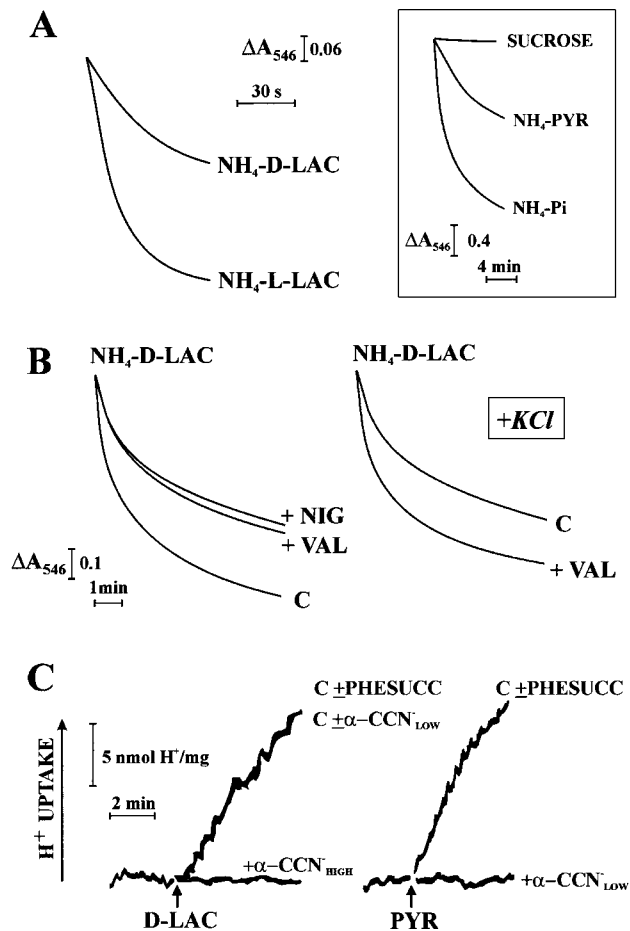
Figure 4   Mitochondrial swelling in ammonium lactate solutions (A and B) and measurements of proton movement across the mitochondrial membrane due to either D-lactate or pyruvate addition to RLM (C)

(**A**) RLM (1 mg of protein) were rapidly added at 25 °C to 2 ml of 0.1 M ammonium phosphate (NH$_4$-Pi), 0.1 M ammonium pyruvate (NH$_4$-PYR) or 0.25 M sucrose (in the inset), 0.1 M ammonium D-lactate (NH$_4$-D-LAC), 0.1 M ammonium L-lactate (NH$_4$-L-LAC) and mitochondrial swelling monitored as described in the Experimental section. (**B**) RLM (1 mg of protein) were added to 2 ml of 0.1 M NH$_4$-D-LAC in the absence (**C**) or presence of 0.5 μg of valinomycin (VAL) or 0.5 nmol of nigericin (NIG) with the swelling monitored as above. Where indicated 25 mM KCl (+ *KCl*) was present. (**C**) RLM (1 mg of protein) were incubated for 3 min at 25 °C in 1.5 ml of the proton medium (see Figure 2B), in the presence of rotenone (2 μg) + antimycin A (1.5 μg) + KCN (1 mM) At the arrows, the following additions were made: D-lactate (D-LAC, 5 mM), pyruvate (PYR, 0.5 mM). Where indicated, either 1 mM or 25 μM αCCN$^-$ (α-CCN$^-_{HIGH}$ or α-CCN$^-_{LOW}$ respectively) or PhSuc (PHESUCC, 10 mM) were added 1 min before D-lactate or pyruvate.

mitochondrial marker enzymes, was checked. The digitonin titration shows that the progressive disruption of the mitochondrial membranes, due to the increasing digitonin/protein ratio, does not allow detection of D-LDH activity. This would be expected in the case of functional and structural association of the enzyme with the mitochondrial inner membrane.

The dependence of the D-lactate oxidation rate on the externally added D-lactate was investigated in ESMP. Saturation kinetics were found with K_m and V_{max} values of 2.9 mM and 52 nmol/min per mg respectively (Figure 3C).

A check was also made of the existence of D-LDH activity in the cytosolic fraction obtained as reported in the Experimental section. The D-LDH activity in cytosol is negligible, as shown by the failure of externally added D-lactate to cause a decrease in the

DCIP absorbance. As a control, cytosolic L-LDH and glucose-6-phosphate dehydrogenase activity was found (results not shown).

D-Lactate can enter mitochondria

The above-reported experiments pose the question as to whether, and how, D-lactate produced in the cytosol can enter mitochondria. To gain a first insight into this point, swelling experiments were carried out. RLM suspended in 0.1 M ammonium D-lactate show spontaneous swelling with a rate and extent lower than that found in ammonium L-lactate, indicating that D- and L-lactate can enter mitochondria and that lactate uptake is stereospecific (Figure 4A). As a control, swelling experiments were carried out using isotonic sucrose, ammonium phosphate and ammonium pyruvate solutions (Figure 4A, inset). As mitochondrial swelling in isotonic salt solutions is due to salt electroneutral entrance into the matrix, we investigated in detail whether D-lactate enters mitochondria in a proton-compensated manner. Thus RLM swelling in ammonium D-lactate solutions was monitored in the presence of certain ionophores under experimental conditions designed to affect selectively either ΔpH or $\Delta\psi$. Decrease in both rate and extent of swelling was found to occur in the presence of nigericin or valinomycin, which cause a decrease in mitochondrial ΔpH [24] (Figure 4B). On the other hand, an increase in both rate and extent was found when swelling was monitored in the presence of valinomycin plus KCl, which causes an increase in mitochondrial ΔpH [24] (Figure 4B). As expected, since both P$_i$ and pyruvate enter mitochondria in a proton-compensated manner, swelling in ammonium phosphate and pyruvate solutions proved to exhibit the same ionophore sensitivity (results not shown). Finally, proton uptake by RLM was found, measured as a function of time following the addition of either 5 mM D-lactate or 0.5 mM pyruvate to mitochondria previously exposed to rotenone, antimycin A and cyanide, i.e. respiratory-chain inhibitors, used to prevent the mitochondrial metabolism (Figure 4C). To find out whether D-lactate transport occurs in a carrier-mediated manner, we checked the sensitivity of the D-lactate-dependent proton uptake to different compounds that cannot enter mitochondria (Figure 4C). In particular, the initial rate of H$^+$ uptake by mitochondria proved to be insensitive to both 10 mM phenylsuccinate (PhSuc), which can inhibit a variety of mitochondrial translocators, including the dicarboxylate and oxodicarboxylate translocators [21], and to 25 μM αCCN$^-$ (αCCN$^-_{LOW}$), a potent inhibitor of the monocarboxylate carrier [37], but was completely inhibited by 1 mM αCCN$^-$. In the same experiment we found that the proton uptake due to 0.5 mM pyruvate addition to RLM was completely prevented by 25 μM αCCN$^-$, but insensitive to 10 mM PhSuc.

Further investigation of D-lactate uptake and metabolism was made by checking the capability of externally added D-lactate to reduce the intramitochondrial dehydrogenase cofactors by means of fluorimetric techniques which have already been used in the case of the nicotinamide nucleotide [23] (Figure 5A), or as developed by us in the case of flavin redox changes (Figures 5B and 5C). No significant reduction of the mitochondrial NAD(P)$^+$ was found as a result of D-lactate addition to RLM previously incubated with the uncoupler FCCP and then treated with rotenone. Surprisingly, in the presence of PhSuc (10 mM), increase of fluorescence was found as result of D-lactate (5 mM) addition. Such an increase was further enhanced by adding sulphite, an inhibitor of the malate dehydrogenase [38], but partially prevented by externally added arsenite (2 mM), which inhibits the pyruvate dehydrogenase complex.

In a control experiment an increase of fluorescence was found which was due to the addition of 0.5 mM pyruvate, which enters
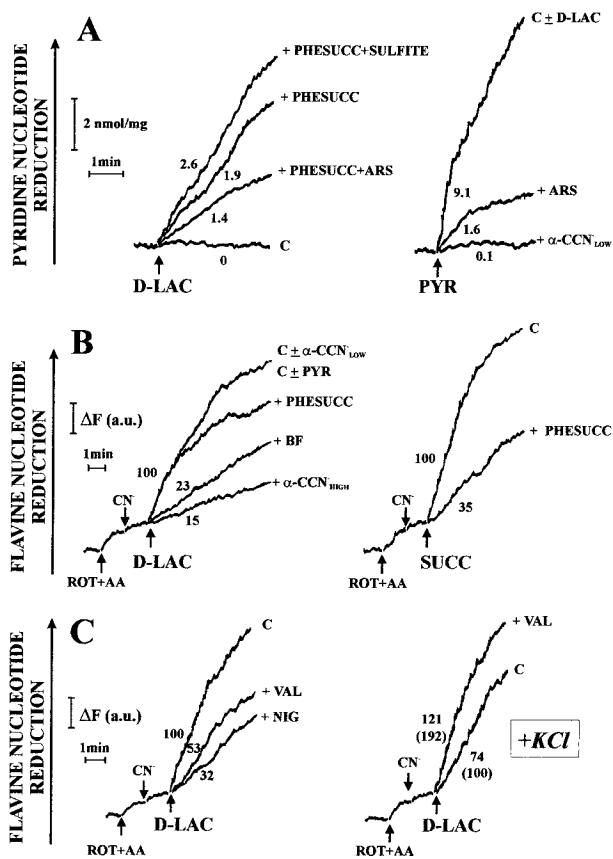
Figure 5   Fluorimetric investigation of the redox state of the mitochondrial pyridine (A) and flavin (B, C) nucleotides caused by externally added D-lactate

(**A**) RLM (1 mg of protein) were incubated at 25 °C for 3–5 min in 2 ml of standard medium in the presence of 1.25 μM FCCP used to oxidize intramitochondrial NAD(P)H. Then rotenone (2 μg) was added after 2 min incubation. At the arrows, either D-lactate (D-LAC, 5 mM) or pyruvate (PYR, 0.5 mM) were added in the absence (**C**) or presence of the following inhibitors (added 1 min before the substrate): PhSuc (PHESUCC, 10 mM), PhSuc (10 mM) plus sulphite (2 mM), PhSuc (10 mM) plus arsenite (ARS, 1 mM), αCCN$^-$ (α-CCN$^-_{LOW}$ 25 μM), ARS (1 mM), D-LAC (5 mM) in the case of pyruvate. Pyridine nucleotide reduction was monitored fluorimetrically ($\lambda_{excitation}$ 334 nm; $\lambda_{emission}$ 456 nm) as a function of time. The rate of fluorescence increase, measured as the tangent to the initial part of the progress curve, is expressed as nmol of intramitochondrial NAD(P)$^+$ reduced/min per mg of mitochondrial protein. For further details, see the Experimental section. (**B**) RLM (1 mg of protein) were incubated for 3 min at 25 °C in 2 ml of standard medium in the presence of 1.25 μM FCCP to oxidize intramitochondrial flavins. Then rotenone (ROT, 2 μg) plus antimycin (AA, 1.5 μg) and KCN (CN$^-$, 1 mM) were added. At the arrows, D-lactate (D-LAC, 5 mM) or succinate (SUCC, 5 mM) were added in the absence (**C**) or presence of the following inhibitors (added 1 min before the substrate): PhSuc (10 mM), BF (50 μM), α-CCN$^-_{LOW}$ (25 μM), α-CCN$^-_{HIGH}$ (1 mM), pyruvate (PYR, 5 mM) in the case of D-lactate. Flavin nucleotide reduction was monitored fluorimetrically ($\lambda_{excitation}$ 450 nm; $\lambda_{emission}$ 520 nm) as a function of time. The rate, measured as the tangent to the initial part of the progress curve, is expressed in arbitrary units/min per mg of mitochondrial protein. For further details, see the Experimental section. (**C**) RLM (1 mg of protein) were incubated for 3 min at 25 °C in 2 ml of standard medium in the presence of 1.25 μM FCCP to oxidize intramitochondrial flavins. Then rotenone (ROT, 2 μg) plus antimycin (AA, 1.5 μg) and KCN (CN$^-$, 1 mM) were added. Where indicated, standard medium was added with 15 mM KCl (+ KCl). At the arrows, D-lactate (D-LAC, 5 mM) was added in the absence (**C**) or presence of the following compounds, added 3 min before the substrate: valinomycin (VAL, 0.5 μg), nigericin (NIG, 0.5 nmol). Flavin nucleotide reduction was monitored as described above.

RLM via the monocarboxylate carrier and is oxidized via the pyruvate dehydrogenase complex, as shown by the inhibition by αCCN$^-$ (25 μM) or by arsenite (2 mM) respectively. Interestingly,
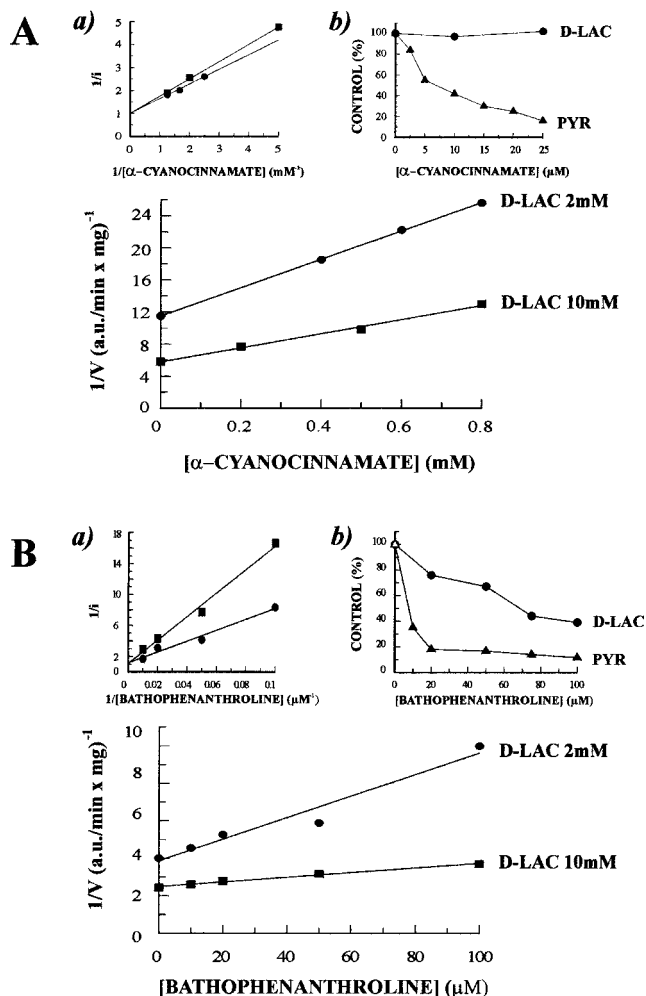
Figure 6   Dixon plot of the inhibition by either αCCN$^-$ or BF of the rate of reduction of intramitochondrial flavin nucleotide due to externally added D-lactate

RLM (1 mg of protein) were incubated at 25 °C in 2 ml of standard medium. The reduction rate of intramitochondrial flavin nucleotide was measured as in Figure 5(B), by using 2 mM (●) and 10 mM D-lactate (■) (in both **A** and in **B**) either in the absence or presence of either αCCN$^-$ (**A**) or BF (**B**) at the indicated concentrations. The rate (v, 'V' on the Figure), measured as the tangent to the initial part of the progress curve, is expressed in arbitrary units/min per mg of mitochondrial protein. The inset (a) in (**A**) and (**B**) is a replot of the data where the fractional inhibition (i) $= 1 - v_i/v_0$; v_i and v_0 are the rates of D-lactate uptake in the presence or absence of the inhibitor. The inset (b) shows the effect of increasing αCCN$^-$ (**A**) or BF (**B**) on either D-lactate (2 mM) or pyruvate (0.2 mM) uptake. The uptake rate values are expressed as percentages of the control value. a.u., arbitrary unit.

no inhibition was found as a result of D-lactate addition (Figure 5A).

In order to find out whether D-lactate (5 mM) can reduce the intramitochondrial flavins, RLM were preincubated with FCCP and then added with rotenone, antimycin A and cyanide (see the Experimental section). This caused a decrease in the flavin fluorescence, i.e. flavin reduction, likely due to endogenous substrate oxidation. When the fluorescence was constant, D-lactate (5 mM) was added. A fast fluorescence decrease occurred with a rate 30 % lower than that measured in the presence of 5 mM succinate in a control experiment. αCCN$^-$ (1 mM) and the metal-complexing agent BF (100 μM) were found strongly to inhibit the rate of flavin reduction; both 25 μM αCCN$^-$ and
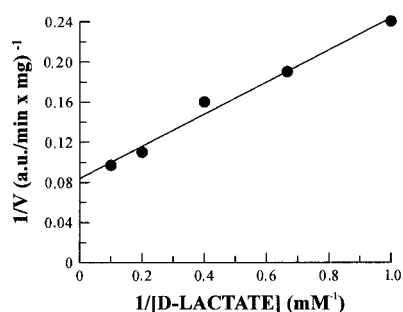
Figure 7   Dependence of the D-lactate uptake rate on increasing concentrations using the double-reciprocal plot

D-Lactate uptake was added to the mitochondrial suspension (1 mg of protein) at the indicated concentrations with the experiment carried out as described in Figure 5(B). The rate (v; 'V' on the Figure), measured as the tangent to the initial part of the progress curve, is expressed in arbitrary units/min per mg of mitochondrial protein. Abbreviation: a.u., arbitrary unit.

5 mM pyruvate did not cause inhibition, while PhSuc was found not to affect the initial rate of fluorescence decrease, even though a significant inhibition occurs 1–2 min after D-lactate addition (Figure 5B). These findings are probably due to D-lactate uptake and intramitochondrial oxidation by the putative flavoprotein D-LDH. In the same experiment, mitochondrial ΔpH and $\Delta\psi$ were changed, as in the swelling experiments, by using valinomycin and nigericin. The rate of the fluorescence decrease, due to D-lactate addition to mitochondria was found to increase by increasing ΔpH and to decrease by decreasing ΔpH as well as in the presence of KCl (Figure 5C). The ionophore sensitivity and the decrease in flavin fluorescence rate by αCCN$^-$ strongly confirm the result of Figure 4(C) and are in favour of the existence of a carrier that transports D-lactate in the matrix in a proton compensated manner, i.e. the D-lactate/H$^+$ symporter.

αCCN$^-$ and BF proved to be non-competitive and competitive inhibitors of the D-lactate-dependent rate of flavin reduction respectively. K_i values were equal to 0.5 mM and 30 μM respectively, as shown by the Dixon plots (Figures 6A and 6B). Interestingly, in both cases the y-intercepts of the lines fitting the experimental points measured in the presence of the inhibitors, which cannot enter mitochondria, coincide with the experimental values measured in the absence of inhibitor. In accordance with the control strength criterion [23], this shows that αCCN$^-$- and BF-sensitive D-lactate transport controls the rate of the measured process, i.e. the rate of the flavin fluorescence decrease reflects the rate of D-lactate transport. The data of Figures 6(A) and 6(B) were plotted as $1/i$ against $1/$[inhibitor], where the fractional inhibition, i, is $1-v_i/v_0$ (see insets a; v_i and v_0 are the rates of D-lactate uptake in the presence or absence of the inhibitor). In both cases, the y-intercepts were 1, showing that both the inhibitors could completely prevent D-lactate uptake. In the same experiment, comparison was made between D-lactate (2 mM) and pyruvate (0.2 mM) with respect to the ability of αCCN$^-$ and BF to prevent their uptake as monitored fluorimetrically as in Figure 5. No inhibition of D-lactate uptake was found due to αCCN$^-$ used up to 25 μM, whereas, at the same concentration, pyruvate uptake showed about 85% inhibition (Figure 6A, inset b). BF proved to inhibit both the reactions, even though with a different sensitivity: 20 μM inhibitor gave 24 and 82% inhibition for D-lactate and pyruvate uptake respectively (Figure 6B, inset b).

The dependence of the rate of D-lactate/H$^+$ symport on increasing D-lactate concentrations was studied by means of a
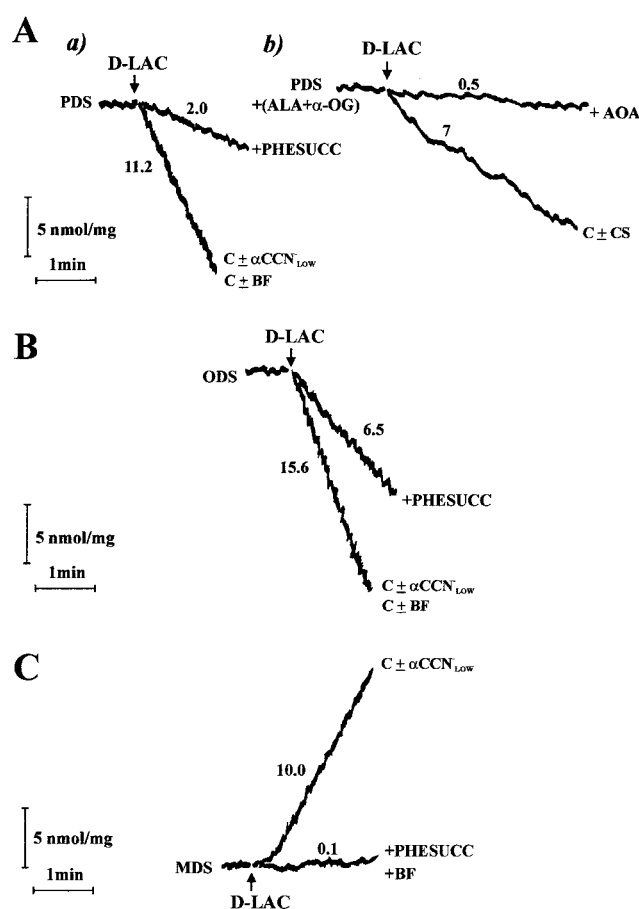

Figure 8   Appearance of pyruvate, malate and oxaloacetate induced by the addition of D-lactate to RLM

RLM (1 mg of protein) were suspended at 25 °C in 2 ml of standard medium in the presence of (**A**) the PDS (0.2 mM NADH plus 1 unit of L-LDH) (*a*) plus rotenone (2 μg), antimycin A (1.5 μg), arsenite (2 mM), alanine (ALA, 1 mM) plus α-oxoglutarate (α-OG, 0.5 mM) (*b*); (**B**) the ODS (0.2 mM NADH plus 2 units of malate dehydrogenase); (**C**) the MDS (0.25 mM NADP$^+$ plus 0.2 unit of 'malic' enzyme). At the arrow, D-lactate (D-LAC, 5 mM) was added in the absence ('C') or presence of the following inhibitors (added 1 min before the substrate): BF (0.1 mM), PhSuc (PHESUCC, 10 mM) or αCCN$^-$ (α-CCN$^-_{LOW}$, 25 μM). In (**A**, *b*), either amino-oxyacetate (AOA, 5 mM) or cycloserine (CS, 5 mM) was added 2 min before the substrate pair alanine + α-oxoglutarate. The values against the traces give the rate of absorbance changes measured as tangents to the initial part of the progress curves and are expressed as nmol of NADH oxidized (NADP$^+$ reduced)/min per mg of mitochondrial protein.

double-reciprocal plot (Figure 7). Saturation characteristics were found, with a K_m value of 1.9 mM.

The addition of D-lactate to RLM causes metabolite appearance in the extramitochondrial phase

To gain further insight into the mechanism of D-lactate uptake into RLM as well as into the physiological role of the mitochondrial D-lactate metabolism, the capability of D-lactate to cause the efflux of several metabolites, newly synthesized in the mitochondrial matrix from the taken up D-lactate, was checked by using specific metabolite detecting systems (see the Experimental section). The appearance of pyruvate, oxaloacetate and malate outside mitochondria was investigated in the same experiment (Figure 8). The pyruvate, oxaloacetate and malate concentrations outside RLM were negligible, since no change in the absorbance measured at 334 nm was found in the presence of
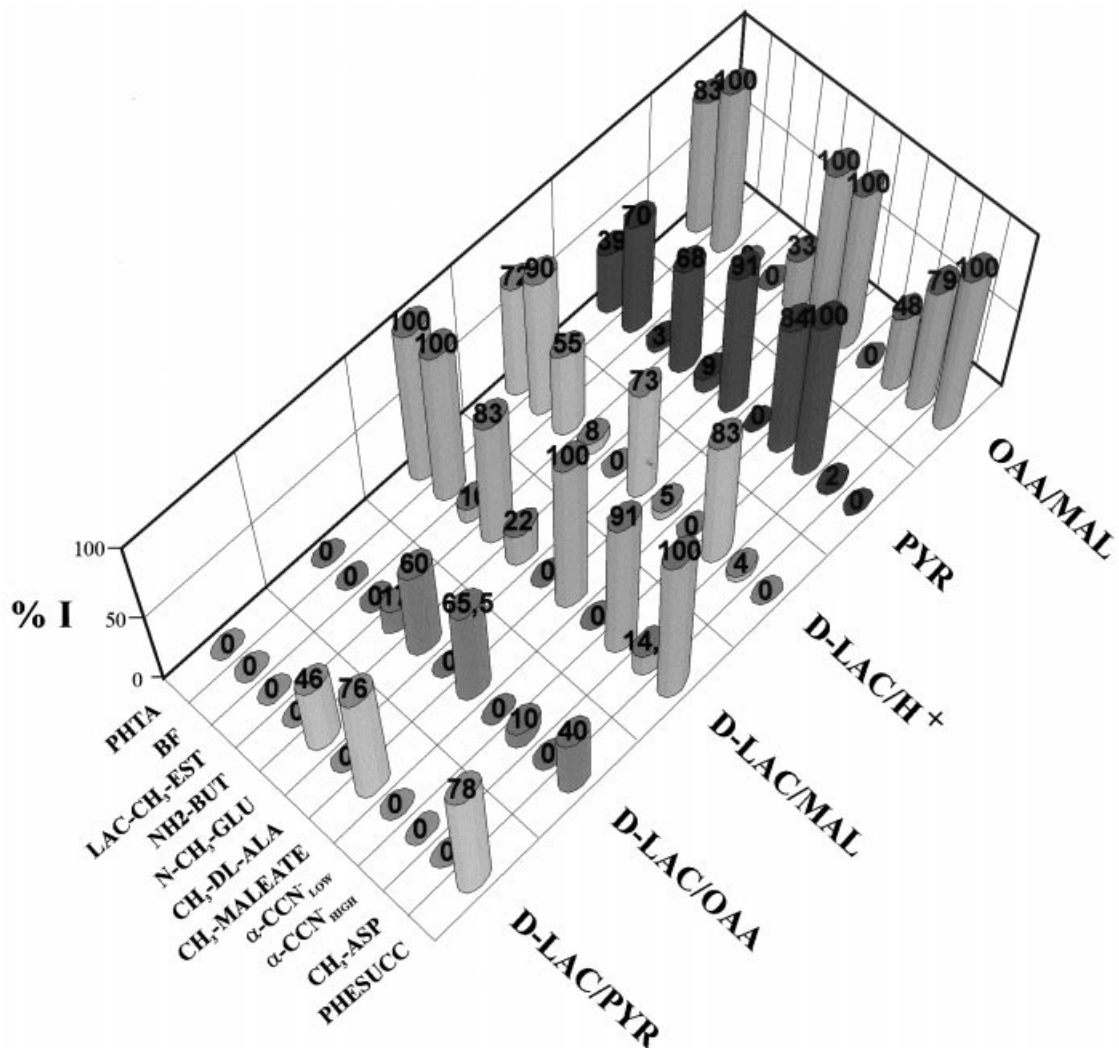
Figure 9   Sensitivity of the D-lactate/pyruvate, D-lactate/oxaloacetate, D-lactate/malate and oxaloacetate/malate antiports and of the D-lactate/H$^+$ and pyruvate/H$^+$ symports to a variety of compounds

The transport processes were measured as described in the Experimental section and in Figures 5 and 8 by using 2 mM D-lactate, 0.2 mM pyruvate and 0.02 mM oxaloacetate, either in the absence or in the presence of the following compounds (added to RLM 1 min before the substrate); phthalonate (PHTA, 1 mM), BF (0.1 mM), lactate methyl ester (LAC-CH$_3$-EST, 10 mM), β-aminobutyrate (NH$_2$-BUT, 10 mM), N-methylglutamate (N-CH$_3$-GLU, 10 mM), methyl-DL-alanine (CH$_3$-DL-ALA, 10 mM), methylmaleate (CH$_3$-MALEATE, 10 mM), αCCN$^-$ (α-CCN$_{HIGH}^-$ or α-CCN$_{LOW}^-$, 1 mM or 25 μM respectively), methylaspartate (CH$_3$-ASP, 10 mM) and PhSuc (PHESUCC, 10 mM).

PDS, ODS and MDS respectively. As a result of 5 mM D-lactate addition, pyruvate (Figure 8A, insert *a*), oxaloacetate (Figure 8B) and malate (Figure 8C) appearance, was found as shown by the decrease of NADH absorbance, in the case of pyruvate and oxaloacetate, and by the increase of the NADPH absorbance in the case of malate. A possible explanation of these findings is that D-lactate, which can enter RLM via the D-lactate/H$^+$ symporter, forms pyruvate, oxaloacetate and malate in the matrix because of the presence of the putative D-LDH, pyruvate dehydrogenase (which provides both CO$_2$ and the allosteric effector of pyruvate carboxylase, acetyl-CoA), pyruvate carboxylase and malate dehydrogenase; these metabolites can, in turn, get the extramitochondrial phase in exchange with further D-lactate.

Interestingly, PhSuc (10 mM) was found to inhibit the appearance of pyruvate, oxaloacetate and malate, whereas BF

(0.1 mM) did not affect pyruvate and oxaloacetate efflux, but completely prevented malate efflux. In any case no inhibition was found to be due to αCCN$^-$ at 25 μM.

In order to check whether pyruvate newly synthesized in the matrix can efflux from mitochondria via free diffusion and/or via the monocarboxylate carrier independently from the presence of external counteranion, RLM, incubated in the presence of PDS, arsenite and the respiratory-chain inhibitors, i.e. rotenone and antimycin, were added with alanine (1 mM) plus 2-oxoglutarate (0.5 mM), thus providing the substrate pair for the mitochondrial alanine aminotransferase to give pyruvate plus glutamate. No change in the NADH absorbance was found, which shows the failure of pyruvate, accumulated in the mitochondria, to efflux spontaneously outside mitochondria (Figure 8A, inset *b*). As a result of D-lactate addition (5 mM), which *per se* cannot cause pyruvate efflux, under these experimental conditions (results not

A

B
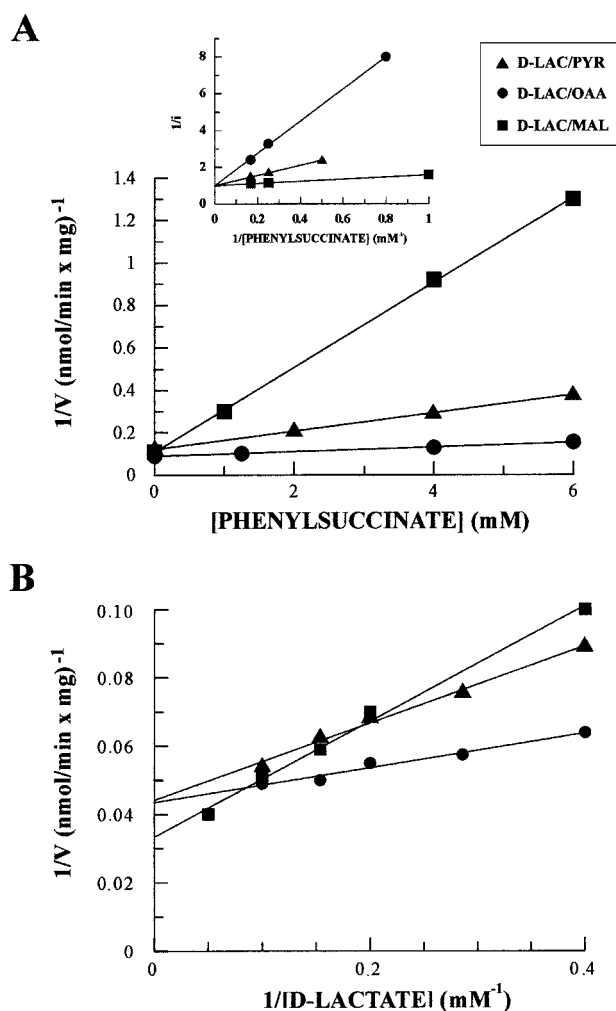
Figure 10   Dependence of the D-lactate/pyruvate, D-lactate/malate and D-lactate/oxaloacetate exchange rates on increasing D-lactate concentrations

(**A**) RLM (1 mg of protein) were suspended in 2 ml of standard medium under the same experimental conditions as those reported in Figure 8. D-Lactate/pyruvate (▲), D-lactate/oxaloacetate (●) and D-lactate/malate (■) exchange rates were measured by using 2.5 mM D-lactate either in the absence or presence of increasing PhSuc concentrations (Dixon plot). The experimental data were also plotted as $1/i$ against 1/PhSuc (inset) where $i = 1 - v_i/v$; v_i and v_0 are the rates of D-lactate/metabolite antiports in the presence or in the absence of the inhibitor. (**B**) D-Lactate was added to the mitochondrial suspension at the indicated concentrations. The exchange rates were measured as tangents to the initial part of the progress curves and expressed as nmol of NADH oxidized (NADP$^+$ reduced)/min per mg of mitochondrial protein.

shown), a decrease in absorbance was found, which indicates the efflux of the intramitochondrial pyruvate. Such an efflux proved to be unaffected by cycloserine (5 mM), but was completely prevented by amino-oxyacetate (5 mM). Both of these compounds are transaminase inhibitors, but they differ from each other as the former cannot enter mitochondria, whereas the latter can. No inhibition of the rate of the pyruvate efflux caused by D-lactate was found in the presence of 25 μM α-CCN$^-$ (results not shown).

The proton-compensated D-lactate uptake, the D-lactate-dependent metabolite effluxes, the oxaloacetate ⇌ malate exchange and the monocarboxylate carrier were compared with respect to their sensitivity to a variety of compounds, including BF, α-CCN$^-$ (either $_{LOW}$ or $_{HIGH}$), phthalonate, PhSuc, methylgluta-
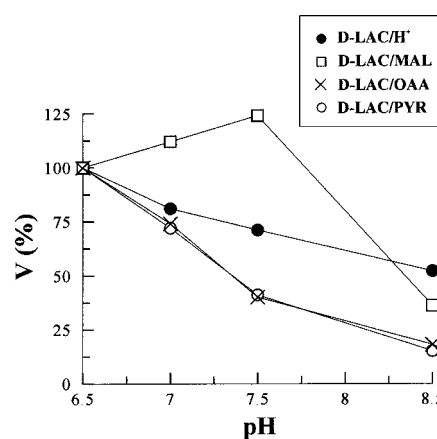
Figure 11   pH profiles of D-lactate transport in RLM

RLM (1 mg of protein) were incubated at 25 °C in 2 ml of standard medium the pH of which was adjusted to the indicated values with either Tris or HCl. Experimental conditions were as in Figure 5(B) for D-lactate (D-LAC)/H$^+$ symport, and in Figure 8 for D-lactate/metabolite antiporters. The D-lactate concentration was 5 mM. The rates were expressed as a percentage of the rate calculated at the pH 6.5 value (taken to be 100%). Abbreviations: MAL, malate; PYR, pyruvate.

mate, lactate methyl ester, methylaspartate, methylmaleate, methyl-DL-alanine, and β-aminobutyrate. The D-lactate, oxalo-acetate and pyruvate concentrations were 2 mM, 20 μM and 0.2 mM respectively, with the inhibitor concentrations varying between 1 and 10 mM, except for BF, which was 0.1 mM (Figure 9). The inhibition profiles show that the appearance of pyruvate and OAA outside RLM added with D-lactate share common features, whereas they differ from the D-lactate-dependent malate efflux and from the proton-compensated D-lactate uptake. All the D-lactate transports were found to differ, with respect to their inhibition sensitivity, from pyruvate uptake and oxaloacetate ⇌ malate exchange.

In any case, the application of the control strength criterion shows that PhSuc-sensitive processes, i.e. the transports across the mitochondrial membrane, are the rate-limiting steps of the absorbance changes. Thus the rate of pyruvate, OAA and malate appearance due to D-lactate is the rate of the transport process (see Figure 10A). Moreover, the fractional inhibition calculated at the infinite PhSuc concentration was found to be 1 (Figure 10A, inset), thus excluding the possibility of spontaneous metabolite efflux from mitochondria.

In a typical experiment, the dependence of the rate of pyruvate, OAA and malate appearance in the extramitochondrial phase on increasing concentration of D-lactate externally added to RLM, photometrically measured under the same experimental conditions, was studied by means of a double-reciprocal plot (Figure 10B). The reactions reveal hyperbolic characteristics. K_m and V_{max} values were 2.5 mM and 23 nmol/min per mg (D-lactate/pyruvate), 1.2 mM and 23 nmol/min per mg (D-lactate/OAA) and 5 mM and 30 nmol/min per mg (D-lactate/malate). In four similar experiments, carried out with different mitochondrial preparations, the V_{max} values of the D-lactate/pyruvate and D-lactate/oxaloacetate exchanges were not different, whereas they differ from the V_{max} of the D-lactate/malate antiport, as judged by a statistical analysis carried out by using Student's t test. The mean V_{max} values were 21 ± 2, 22 ± 1 and 30 ± 1.5 nmol/min per mg of protein ($P < 0.02$); the mean K_m values were 2.4 ± 0.2, 1.28 ± 0.2 and 5.1 ± 0.4 mM respectively.

The pH profiles of the efflux of pyruvate, malate and oxalo-acetate were also investigated (Figure 11). The rate of malate efflux caused by 5 mM D-lactate was found to remain fairly constant over the pH range 6.5–7.5, whereas both pyruvate and OAA efflux was found to decrease with increasing pH with no significant difference from each other. In the same experiment, the D-lactate/H$^+$ symport was also investigated fluorimetrically as a function of pH, and about 50 % decrease was found over the pH range 6.5–8.5. The FAD/FMN fluorescence constants were found to remain fairly constant under the same experimental conditions.

DISCUSSION

We show that RLM can take up externally added D-lactate, metabolize it in the matrix and export newly synthesized meta-bolites outside the mitochondria. This occurs because of the existence of the D-lactate/H$^+$ symporter, the putative D-LDH located in the inner side of the mitochondrial inner membrane that gives pyruvate, the D-lactate/oxoacid (which mediates both D-lactate $\rightleftharpoons$ pyruvate and D-lactate $\rightleftharpoons$ OAA exchanges) anti-porter and D-lactate/malate antiporter.

D-Lactate metabolism and transport in RLM is discussed separately below.

The existence of the putative mitochondrial D-LDH

We show that RLM can oxidize externally added D-lactate because of the existence of the putative D-LDH. A D-LDH assay in RLM, mitoplasts and ESMP shows that the putative D-LDH activity is located in the inner side of the mitochondrial inner membrane, thus indicating that D-lactate oxidation occurs in the matrix compartment. The putative D-LDH is shown to be a flavoprotein capable of reducing Complex III, as suggested by a P/O ratio equal to 2 and, more importantly, by the reduction of the intramitochondrial flavins in the presence of rotenone as fluorimetrically determined.

In this regard, RLM putative D-LDH is similar to the D-LDHs found in *Saccharomyces cerevisiae* mitochondria [34,39] and in *Escherichia coli* [40].

The inside localization accounts for the mitochondrial oxid-ation of the D-lactate newly synthesized in the matrix from lactoylglutathione by mitochondrial glyoxalase II [17–20], as well as for the cytosolic D-lactate taken up by mitochondria. In any case, the occurrence of the novel putative D-LDH accounts for the removal of methylglyoxal, which is toxic to the liver [6].

Since apparently no D-lactate oxidation takes place in the cytosolic fraction (see the Results section), we conclude that D-lactate oxidation is a mitochondrial process. At present, we have no information on the characteristics of D-LDH. However, we can distinguish it from the L-LDH which oxidizes the L isomer in a NAD(P)-dependent manner, and on the basis of the lack of inhibition of the putative D-LDH by oxamate (results not shown) [4,5]. As expected, in the light of the occurrence of two different mitochondrial LDHs, we could not find any lactate racemase activity in RLM, as shown by the failure to reveal fluorimetrically L-lactate formation in both coupled RLM and extracts of RLM, previously incubated with D-lactate (results not shown).

The D-lactate symporter

In order to study D-lactate uptake by RLM, use was made of spectroscopic techniques under conditions in which mito-chondrial metabolism is mostly active, thus allowing for moni-toring mitochondrial reactions and traffic of the newly synthesized substrates across the mitochondrial membrane. In particular, D-lactate/H$^+$ symport was investigated with a novel method that allows for measurements of the D-lactate transport by changes in intramitochondrial flavin fluorescence. The D-lactate/pyruvate, D-lactate/OAA and D-lactate/malate antiports were studied photometrically by using the metabolite-detecting systems already used (see [22–25]). In any case, control-strength-criterion application shows that the measured change of both fluorescence and absorbance mirrors the rate of substrate trans-port. Thus if the *in vivo* situation is similar to the *in vitro* situation, D-lactate metabolism should be regulated by transport processes.

That mitochondria can take up D-lactate with net carbon uptake in a proton-compensated manner is shown by swelling in isotonic ammonium D-lactate solution which, *per se*, indicates that D-lactate uptake occurs in a proton-compensated manner. Such a conclusion is strongly substantiated by the correlation found between (i) ΔpH and swelling rate, (ii) ΔpH and D-lactate uptake measured as flavin fluorescence decrease and (iii) by proton uptake by mitochondria in the presence of D-lactate, under conditions in which D-lactate oxidation is completely prevented. One could argue that this result is not consistent with the ΔpH-dependence of the uptake process, but the residual ΔpH could still drive the substrate movement [24].

The existence of a carrier that mediates D-lactate/H$^+$ symport in RLM is shown by hyperbolic kinetics of the transport and from the inhibition studies. Since D-lactate uptake by RLM can also occur in exchange with pyruvate, OAA and malate, we could consider the rate of flavin fluorescence changes as reflecting the activity of both D-lactate symporter and antiporters. How-ever, the failure of externally added PhSuc to inhibit the initial rate of flavin reduction rules out the possibility that we measure fluorimetrically the D-lactate uptake via the two antiporters. Unfortunately we cannot calibrate the fluorimetric response with certainty, since the fluorescence yield depends on the nature of the fluorophore. However, by assuming that the fluorescence constants of the flavins contained in the succinate dehydrogenase complex and in the putative D-LDH are similar and that the maximum rate of the succinate-dependent change of fluorescence is equal to the V_{max} of the succinate-dependent oxygen uptake [41], we calculated that the V_{max} of the D-lactate proton-compensated uptake is at least about 70 nmol/min per mg of protein. The measured rate of proton uptake due to D-lactate is about 8 nmol/min per mg of protein, a value lower than that calculated. However, this could be due to the different media used. Consistently a reduction in the rate of flavin fluorescence decrease occurred in the presence of Na$^+$.

The competitive inhibition by BF, which inhibits the rate of D-lactate uptake by virtue of its ability to bind metal ion(s), as shown by the prevention and removal experiments carried out as in [42,43], but not shown in detail, is in favour of the existence of a metal ion(s) at the substrate-binding site. Such a conclusion is not unique, since dicarboxylate, tricarboxylate and oxo-dicarboxylate translocators show the same nature of inhibition by BF [42]. On the other hand, the failure of BF to inhibit the P$_i$ carrier [42] rules out the possibility that this carrier can transport D-lactate. Interestingly, the D-LDH in *Archaeoglobus fulgidus* proved to be a Zn^{2+}-containing enzyme [44]. Although at present we cannot exclude with certainty the possibility that the D-lactate/H$^+$ symporter does not translocate the L-isomer, the swelling stereospecificity suggests the occurrence of a different manner of uptake. Moreover, as a result of the combined action of the D-lactate/H$^+$ symporter, the mitochondrial D-LDH, the D-lactate/oxo acid antiporter and the cytosolic L-LDH, L-lactate might derive from D-lactate in the cytosol.
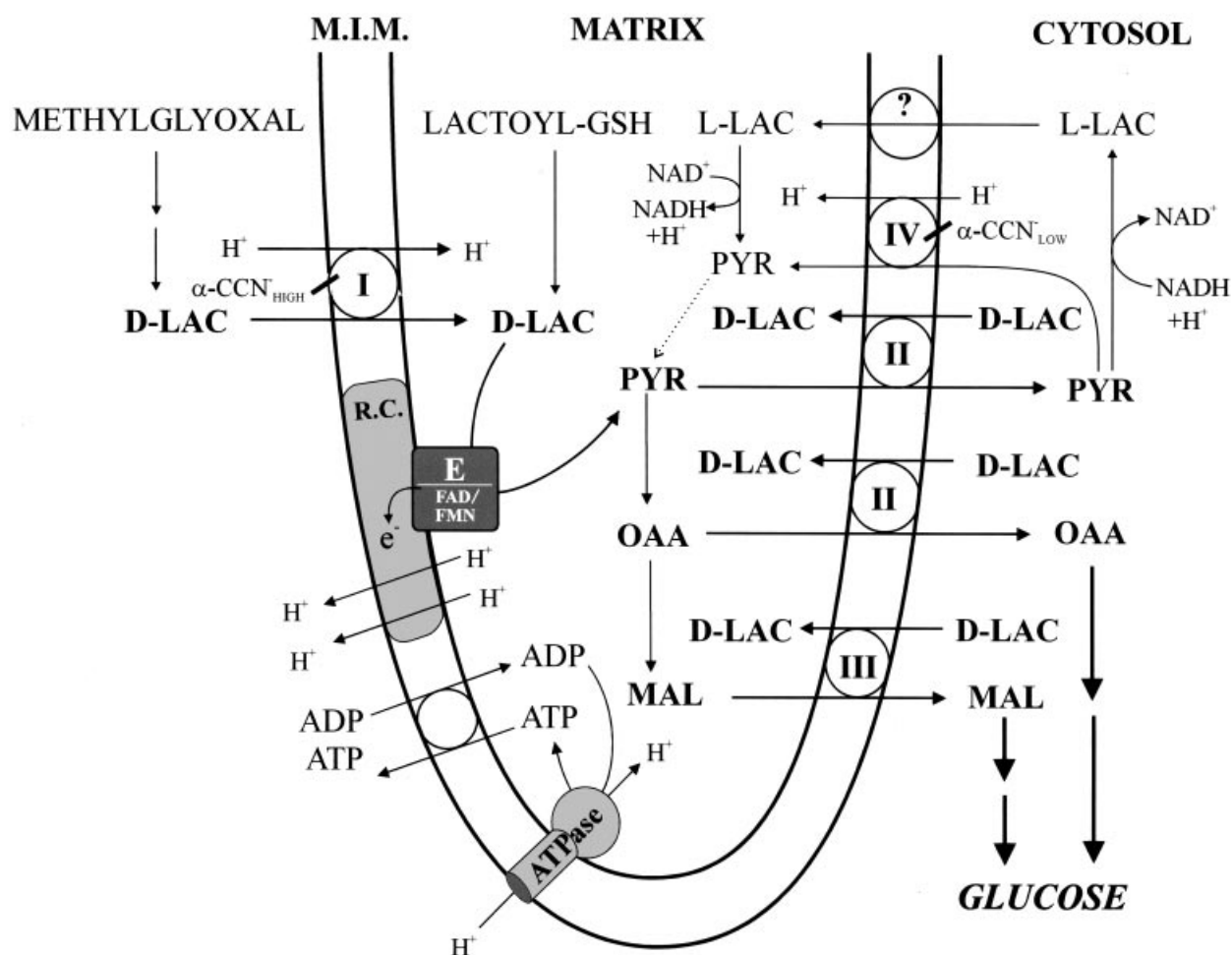
Scheme 1 D-**Lactate transport and metabolism in RLM**

For an explanation, see the text. Abbreviations: $E_{FAD/FMN}$, putative mitochondrial D-LDH; D-(L-)LAC, D-(L-)lactate; LACTOYL-GSH, *S*-D-lactoylglutathione; MAL, malate; PYR, pyruvate; M.I.M., mitochondrial inner membrane; carriers: **I**, D-lactate/H$^+$ symporter; **II**, D-lactate/oxoacid antiporter; **III**, D-lactate/malate antiporter; **IV**, monocarboxylate transporter; **?**, L-lactate transporter; R.C., respiratory chain.

D-Lactate antiporters

The saturation kinetics observed in the case of the pyruvate, OAA and malate efflux, under conditions that reflect the transport step of the measured process, show that the effluxes occur in a carrier-mediated manner (Figure 10). Differently from OAA and malate, pyruvate can cross the mitochondrial membrane in a proton-compensated manner via the monocarboxylate carrier; thus one could argue that pyruvate can efflux from the mitochondrial matrix *per se* via the monocarboxylate carrier, with independent uptake of D-lactate, perhaps via free diffusion and/or via the monocarboxylate carrier. The experimental findings reported here rule out the possibility that both D-lactate uptake and D-lactate metabolite efflux can occur via free diffusion. In fact the calculated fractional inhibition, $i = 1 - (v_i/v_0)$, proved to be 1 as measured at infinite inhibitor concentration (Figures 6A and 6B; Figure 10A). This is not consistent with substrate traffic across the mitochondrial membrane occurring without carrier proteins. On the other hand we show that pyruvate-loaded RLM cannot export pyruvate in the absence of externally added D-lactate (Figure 8).

The conclusion that the monocarboxylate carrier cannot transport D-lactate derives from inhibition studies and is directly shown in Figure 1(C), where in the presence of 25 μM α-CCN$^-$ oxygen uptake occurs with D-lactate, but not with pyruvate as a respiratory substrate.

The D-lactate symporter and antiporters differ from each other and from other mitochondrial carriers

In the light of the differences found in the V_{max} values and, more importantly, in the inhibition and pH profiles (Figures 9 and 11), we conclude that the three D-lactate carriers are separate.

The three D-lactate carriers can be also distinguished from others already shown in RLM, including the dicarboxylate, the tricarboxylate, and the oxoglutarate carriers, in the light of their inhibitor-sensitivity and pH profile. Moreover, we can exclude the possibility that the efflux of oxaloacetate and malate is a result of the combined action of the D-lactate/pyruvate and of the pyruvate/oxaloacetate [22] and pyruvate/malate [28] antiporters, since the V_{max} of the pyruvate/oxaloacetate and pyruvate/malate carriers in RLM are very low, and are not consistent with those measured in the present study.

The existence of different carriers for the same metabolite, only one of them producing net carbon uptake, is not unique.

Both malate and fumarate can enter mitochondria in exchange with P_i as well as with other dicarboxylates [45,46]. Proline uptake in rat kidney mitochondria occurs via the proline uniporter and the proline/glutamate antiporter [24,47]. The occurrence of glutamine uniporter, and of glutamine/glutamate and glutamate/malate antiporters [23] has already been shown. Riboflavin net uptake and antiport with intramitochondrial FAD has been proposed in the riboflavin/riboflavin derivatives cycle [26]. Pyruvate can enter RLM *per se*, but also in exchange with OAA [22], and less efficiently with malate [28]. Thus we suggest that most of the transport processes between cytosol and mitochondria requires the co-operation of uniport/symporters and antiporters. The former provide substrates in the mitochondrial matrix where the taken up substrates are metabolized, and the newly synthesized compounds can either be metabolized in mitochondria, or reach the cytosol to accomplish other processes by using the antiporters.

As far as the energy-dependence of the transport is concerned, the D-lactate transport processes here reported are ΔpH-dependent, either directly or because they depend on the primary transport process.

Metabolic role of D-lactate transport

The picture emerging from the present study is as shown in Scheme 1. Since methylglyoxal is toxic, it must be metabolized to D-lactate [6], which enters mitochondria where the putative D-LDH activity is located (Figure 3). D-Lactate uptake by RLM occurs in a proton-compensated manner (Figures 4 and 5) via the D-lactate/H^+ symporter (Figures 6 and 7). In the matrix, the taken-up D-lactate, as well as D-lactate formed by the mitochondrial glyoxalase II [17–20], is oxidized to pyruvate via the putative flavoprotein D-LDH (Figure 3). Electrons flow to oxygen, producing energy and ATP synthesis (Figures 1 and 2). The fate of the newly synthesized pyruvate could be both mitochondrial and cytosolic. In the matrix, pyruvate metabolism, via pyruvate dehydrogenase and the pyruvate carboxylase, gives OAA, reduced to malate via malate dehydrogenase, with concomitant oxidation of the NADH produced in the pyruvate oxidative decarboxylation. The failure of D-lactate to reduce the intramitochondrial nicotinamide nucleotides is consistent with this explanation, as is NAD(P)H formation under conditions in which pyruvate efflux is impaired by PhSuc (Figure 5). Consistently the inhibition of the malate dehydrogenase by sulphite makes available more pyruvate for its own metabolism with consequent increase in the NAD(P)H formation in RLM (Figure 5). The fate of pyruvate outside mitochondria could be to re-enter mitochondria via its own carrier or to oxidize the extramitochondrial NADH, thus participating in the intracellular lactate shuttle recently proposed [4].

The efflux of OAA and, more importantly, of malate, outside mitochondria (Figure 8) could account for the gluconeogenesis from D-lactate already reported [15]. Consistently, we have found D-lactate uptake and D-lactate-dependent OAA and malate efflux in rat kidney mitochondria, but not in rat heart mitochondria, in which no gluconeogenesis occurs.

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