Partial reconstruction of *in vitro* gluconeogenesis arising from mitochondrial L-lactate uptake/metabolism and oxaloacetate export via novel L-lactate translocators

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In the light of the occurrence of L-lactate dehydrogenase inside the mitochondrial matrix, we looked at whether isolated rat liver mitochondria can take up and metabolize L-lactate, and provide oxaloacetate outside mitochondria, thus contributing to a partial reconstruction of gluconeogenesis *in vitro*. We found that: (1) L-lactate (10 mM), added to mitochondria in the presence of a cocktail of glycolysis/gluconeogenesis enzymes and cofactors, can lead to synthesis of glyceraldehyde-3-phosphate at a rate of about 7 nmol/min per mg mitochondrial protein. (2) Three novel translocators exist to mediate L-lactate traffic across the inner mitochondrial membrane. An L-lactate/H^+ symporter was identified by measuring fluorimetrically the rate of endogenous pyridine nucleotide reduction. Consistently, L-lactate oxidation was found to occur with P/O ratio $= 3$ (where P/O ratio is the ratio of mol of ATP synthesized to mol of oxygen atoms reduced to water during oxidative phosphorylation) and with generation of membrane potential. Proton uptake, which occurred as a result of addition of L-lactate to RLM together with electron flow inhibitors, and mitochondrial swelling in ammonium L-lactate solutions were also monitored. L-Lactate/oxaloacetate and L-lactate/pyruvate antiporters were identified by monitoring photometrically the appearance of L-lactate counter-anions outside mitochondria. These L-lactate translocators, which are distinct from the monocarboxylate carrier, were found to differ from each other in V_{max} values and in inhibition and pH profiles, and proved to regulate mitochondrial L-lactate metabolism *in vitro*. The role of lactate/mitochondria interactions in gluconeogenesis is discussed.

Key words: antiporter, dehydrogenase, glyceraldheyde-3-phosphate, symporter, transport.

INTRODUCTION

Although L-lactate production in skeletal muscles has been studied for nearly two centuries, its metabolism and functional role is still a matter of debate (see [1]). The transport of L-lactate in and out of cells is facilitated by one of the 14 monocarboxylate transporters [2], while lactate clearance has been attributed both to L-lactate oxidation in muscle, with organ/cell specific features, (see [3]) and to gluconeogenesis (GNG) in liver and kidney. In liver cytosol, L-lactate is oxidized to pyruvate via the cytosolic L-lactate dehydrogenase (L-LDH), thus starting GNG, with a major role proposed for mitochondrial aspartate/glutamate antiport to export carbon chains [4]. Given that the occurrence of two mitochondrial L-LDH has been reported by several authors [5–8], even though such a report has been questioned [9,10], reinvestigation of GNG from L-lactate is needed, with a particular focus on the role played by mitochondria in such a process. Indeed, as distinct from the position with D-lactate [11], whether and how L-lactate uptake by rat liver mitochondria (RLM), and whether and how mitochondrial L-lactate metabolism can contribute to GNG, remains to be established.

In this study we investigated whether metabolism of externally added L-lactate can provide GNG substrates in the extramitochondrial phase. We show that, as a result of addition of L-lactate to

RLM, glyceraldehyde-3-phosphate (G-3-P) is synthesized outside mitochondria in the presence of most of the glycolysis/GNG enzymes and cofactors. This is due to the combined action of the matrix L-LDH, which allow for energy production and GNG precursor synthesis, and of two novel carriers [L-lactate/H^+ symporter and L-lactate/OAA (oxaloacetate) antiporter] which allow L-lactate uptake by RLM in symport with a proton, and the efflux of OAA newly synthesized as a result of L-lactate mitochondrial metabolism. The occurrence of an L-lactate/pyruvate antiporter, which accounts for reducing equivalent transfer into mitochondria via a shuttle, is also shown [12].

EXPERIMENTAL

Materials

Rabbit muscle L-LDH (EC 1.1.1.27), chicken liver malic enzyme (ME; EC 1.1.1.40), corn phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.31), Baker's yeast enolase (EC 4.2.1.11), rabbit muscle phosphoglycerate mutase (PGM) (EC 5.4.2.1), yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), yeast 3-phosphoglyceric phosphokinase (PGK; EC 2.7.2.3), rabbit muscle aldolase (EC 4.1.2.13), NADH, $NADP^+$, rotenone, antimycin A, mixothiazole (MIXO), FCCP

(carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), sodium sulphite, valinomycin, nigericin, KCl, mannitol, TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine), GTP, ATP, ADP, dihydroxyacetone phosphate, D-lactic, L-lactic, succinic, malic, glutamic, *N*-methylglutamic, DL-*β*-amino-n-butyric, *N*-methylaspartic, *α*-cyano-4-hydroxycinnamic and ascorbic acids, bathophenanthroline and *N*-methyl-DL-alanine were obtained from Sigma; methylmaleic and phenylsuccinic acids were obtained from Fluka (Gillingham, Dorset, U.K.). Phthalonic acid was prepared as described previously [13]; pig heart malate dehydrogenase (MDH; EC 1.1.1.37), sucrose, Triton X-100 (TX-100), Hepes, Tris and sodium arsenite were from J. T. Baker (Noisy-le-Sec, France).

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, MIXO and FCCP were dissolved in ethanol.

Isolation of RLM

RLM were isolated from male Wistar rats (150–200 g) as described previously [14], using a medium consisting of 0.25 M sucrose, 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. Mitochondrial protein was measured as described previously [15]. In the light of the reported ability of cytosolic L-LDH to bind to mitochondria [16], we checked whether its presence in our mitochondrial preparation could contribute in some way to metabolism of either added lactate or effluxed metabolites.

We found that in the presence of an inhibitor cocktail including inhibitors of the respiratory chain (rotenone, antimycin A and cyanide) and the transport inhibitor phenylsuccinate, addition of pyruvate (1 mM) caused oxidation of NADH (0.2 mM) at a rate equal to 0.9 ± 0.01 μmol/min per mg protein in three experiments, whereas negligible oxidation of NADH was found when the pyruvate concentration was 20 μM. No NADH formation occurred when 10 mM L-lactate plus 1 mM NAD^+ were added to mitochondria.

GNG partial reconstruction system (GPRS)

Appearance of G-3-P was monitored photometrically, at 25 °C, by means of a PerkinElmer Lambda-5 spectrophotometer, following the absorbance decrease at 334 nm due to NADH (0.2 mM) oxidation, which occurs in the presence of the GPRS consisting of: PEPCK (0.1 unit), enolase (2 units), PGM (2 units), PGK (2 units), GAPDH (2 units), GTP (1 mM), ATP (1 mM), and rabbit muscle aldolase (1 unit), plus dihydroxyacetone phosphate (1 mM), used to displace to the reaction to the right. Briefly, mitochondria (1 mg of protein) were incubated in 2 ml of a standard medium, consisting of 0.2 M sucrose, 10 mM KCl, 20 mM Hepes/Tris, pH 7.2, 1 mM $MgCl_2$, and NADH (0.2 mM) both in the sample and in the reference cuvette in which GPRS, and GPRS minus GAPDH, were also present respectively. L-Lactate (10 mM) was then added simultaneously to both cuvettes and NADH oxidation was measured. The rate of absorbance change was obtained as the tangent to the initial part of the progress curve and expressed as nmol of NADH oxidized/min per mg of mitochondrial protein. The ε_{334} value measured for NADH was found to be 6.5 $mM^{-1} \cdot cm^{-1}$. It was also verified that the compounds used in this work, including the transport inhibitor as phenylsuccinate, had no effect on the GPRS enzyme reactions.

When measuring the amount of newly synthesized G-3-P, L-lactate was added to RLM plus GPRS minus NADH, and L-lactate metabolism was blocked by addition of rotenone (2 μg),

antimycin A (1.5 μg) and cyanide (1 mM). Then NADH (0.2 mM) was added and the amount of G-3-P synthesized outside RLM was calculated from the absorbance decrease.

L-LDH assay

The L-LDH assay was performed photometrically by means of a PerkinElmer Lambda-5 spectrophotometer, at 600 nm and at 25 °C, as described previously [11,17].

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 $MgCl_2$, and 5 mM KH_2PO_4/K_2HPO_4 using a Gilson 5/6 oxygraph and a Clark electrode. Since L-lactate-dependent oxygen consumption occurs slowly, L-lactate was added to RLM incubated in the oxygraph vessel after complete oxidation of the endogenous substrates had occurred (approx. 2 min).

Assay of the safranine response

The safranine O response was monitored as described in [18,19]. Measurements were carried out at 25 °C in 2 ml of RLM isolation medium containing 9.6 μM safranine O and 1 mg mitochondrial protein.

Swelling experiments

Mitochondrial swelling at 25 °C was followed, 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 (pH was adjusted to 7.2), and the decrease in the absorbance was continuously recorded.

Measurement of proton movement

Proton movement across the mitochondrial membrane was followed, as described previously [18,19], using a Gilson 5/6 Oxygraph, equipped with a Gilson pH 5 Servo Channel electrode which allows for continuous monitoring and direct recording pH changes in the mitochondrial suspension 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 assays

Changes in the redox state of either flavin or pyridine nucleotides was monitored fluorimetrically, using a PerkinElmer luminometer LS-5 with excitation and emission wavelengths of 450–520 nm and 334–456 nm respectively. L-Lactate and pyruvate uptake was monitored as described in [11,20]. The NAD(P)H fluorescence was calibrated as described in [21–23].

Appearance of pyruvate, malate and OAA outside mitochondria, caused by externally added L-lactate, was monitored by using the pyruvate detecting system (PDS; consisting of 200 μM NADH plus 1 unit of L-LDH), the OAA detecting system (ODS; consisting of 200 μM NADH plus MDH, 1 unit) and the malate detecting system (MDS; consisting of 200 μM $NADP^+$ plus ME, 0.2 unit) and then following NADH oxidation photometrically in the case of either pyruvate or OAA, and $NADP^+$ reduction in the case of malate. L-Lactate on its own proved to have no effect on the enzymatic reactions and on A_{334}. 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}.

It was confirmed, using the controls described below, 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, controls were made to test the intactness of the mitochondrial inner membrane by checking the rate of NADH oxidation in the absence of any substrate. When the rate was higher than 5 % of that measured following metabolite efflux, mitochondria were not used. To ensure that NADH oxidation, either spontaneous or induced by other compounds effluxed from mitochondria, could not affect the quantitative measurements in each experiment, NADH was added both in the sample and in the reference cuvette. The rate of NADH oxidation was then measured by difference thus ensuring that the rate of absorbance change was due only to reactions outside the mitochondria (see [24]). When measuring the appearance of OAA outside the mitochondria, L-lactate was also added both to the reference and sample cuvettes, thus allowing for any NADH oxidation occurring due to pyruvate efflux or to contamination by L-LDH. The rates of both fluorescence and absorbance changes were obtained as tangents to the initial parts of the progress curve and expressed as arbitrary units of FAD/FMN reduced/mg of mitochondrial protein and as nmol NADH oxidized (NADP$^+$ reduced)/mg mitochondrial protein respectively.

Statistical analysis and computing

Data are reported as means $\pm$ S.E.M. for the indicated experiments. Statistical analysis was carried out using the Student's t test when necessary. Experimental plots were obtained using Grafit (Erithacus Software, Horley, Surrey, U.K.).

RESULTS

Partial reconstruction of *in vitro* GNG

In order to find out whether externally added L-lactate can itself start GNG independently from its oxidation to pyruvate outside RLM, i.e. to discover whether L-lactate can provide glucose precursor/s in the cell cytosol by a pathway including its mitochondrial transport and metabolism, we measured G-3-P synthesized *in vitro* due to L-lactate added to mitochondria in the presence of the GPRS. The latter allows G-3-P synthesis from both OAA and from the other common glycolysis/GNG metabolites (see Experimental section and the scheme in Figure 1). In controls (results not shown), we found that the absorbance of added NADH remained constant in the presence of both RLM plus L-lactate, and of RLM plus GPRS, thus showing that the mitochondria were intact as well as demonstrating the absence of OAA and substrates of the common glycolysis/GNG pathway in the sample respectively. In the absence of mitochondria, no NADH oxidation was found due to the GPRS component, thus demonstrating further the absence of any gluconeogenetic substrate.

As a result of addition of L-lactate (10 mM), NADH oxidation occurred at a rate of 7.2 nmol/min per mg (7.0 ± 0.5 in three experiments) (Figure 1A). Externally added phenylsuccinate (10 mM), which can inhibit a variety of mitochondrial translocators [25], but not externally added enzymes was found to cause about 40 % inhibition. Consistently, when RLM plus GPRS were incubated for 5 min with L-lactate, followed by addition of rotenone, antimycin and cyanide to block metabolism, subsequent addition of NADH showed that 36.5 nmol of glyceraldehyde had been synthesized. This shows that G-3-P synthesis proceeded linearly for at least for 5 min. Detection of the product required

the presence of GAPDH (Figure 1B), and was totally prevented by addition of the above inhibitors before the addition of L-lactate (results not shown).

L-Lactate transport and oxidation by RLM

The above findings pose the question as to how L-lactate can enter mitochondria and cause efflux of OAA or other metabolites, thereby starting GNG in the extramitochondrial phase. In order to gain a first insight into this point, as well as into the matrix L-LDH reaction, investigations of L-lactate uptake and metabolism were made by checking the capability of externally added L-lactate to reduce the intramitochondrial dehydrogenase cofactors. This was done by using fluorimetric techniques which can reveal either flavin [11] or pyridine nucleotide redox changes [20,21]. In contrast with the situation with D-lactate [11], addition of L-lactate to RLM caused no reduction of the mitochondrial flavins (results not shown). Reduction of the intramitochondrial NAD(P)$^+$ was studied in RLM previously preincubated with FCCP, added to allow oxidation of endogenous NAD(P)H, followed by rotenone to prevent the oxidation of the newly formed NAD(P)H (see the Experimental section). When the fluorescence had stabilized, L-lactate (5 mM) was added and was found to cause a fast fluorescence increase due to reduction of NAD(P)$^+$ (Figure 2). Phenylsuccinate (10 mM) and phthalonate (1 mM), both carrier inhibitors that cannot enter RLM (see [25]), were found to inhibit the initial rate of L-lactate-dependent NAD(P)H formation, whereas both 25 μM α-cyano-4-hydroxycinnamate (α-CCN$^-$), a potent inhibitor of the monocarboxylate carrier [26], and methylmaleate (10 mM) were ineffective in reducing the initial rate of reaction, even though a significant inhibition was observed 30 s after addition of L-lactate. As a control, we checked that pyruvate oxidation was blocked by 25 μM α-CCN$^-$ (Figure 2, inset). Phenylsuccinate proved to be a competitive inhibitor ($K_i = 8$ mM) of the rate of NAD(P)$^+$ reduction (Figure 3A). Interestingly, the y-intercepts of the lines fitting the experimental points obtained in the presence of the inhibitor, coincide with the experimental value obtained in the absence of inhibitor. In accordance with the control strength criterion [25,26], this shows that phenylsuccinate-sensitive L-lactate transport controls the rate of the measured process, i.e. the rate of the NAD(P)H fluorescence increase reflects the rate of the L-lactate transport. In order to confirm such a conclusion, as in [22], the detergent TX-100 was added in the presence of NADP$^+$ (1 mM) to RLM pre-incubated with L-lactate; following a rapid decrease of fluorescence due to the mitochondria solubilization, the rate of fluorescence change was found to increase, thus confirming that the measurement of fluorescence change rate reflects the rate of L-lactate transport (results not shown).

The data from Figure 3(A, bottom panel), were also plotted as $1/i$ against $1/$[phenylsuccinate], where the fractional inhibition, i, is $1 - V_i/V_o$ (Figure 3A, top panel). The y-intercept was unity, showing that the phenylsuccinate can prevent L-lactate uptake completely.

The dependence of the rate of L-lactate transport on increasing L-lactate concentrations was analysed using a Michaelis–Menten plot (Figure 3B). Saturation characteristics were found with K_m and V_{max} values equal to 3.5 mM and 22 nmol/min per mg protein respectively. In reasonable agreement with Figure 2, we found oxygen consumption due to addition of 5 mM L-lactate to RLM to occur with a rate of 9 ng-atoms of O/min per mg; as expected, oxygen consumption was inhibited by rotenone, due to the mitochondrial NAD$^+$-dependent L-LDH. As a control, D-lactate (5 mM), which is oxidized by the flavoprotein D-LDH [11] was found to restore respiration, in a manner that was totally inhibited
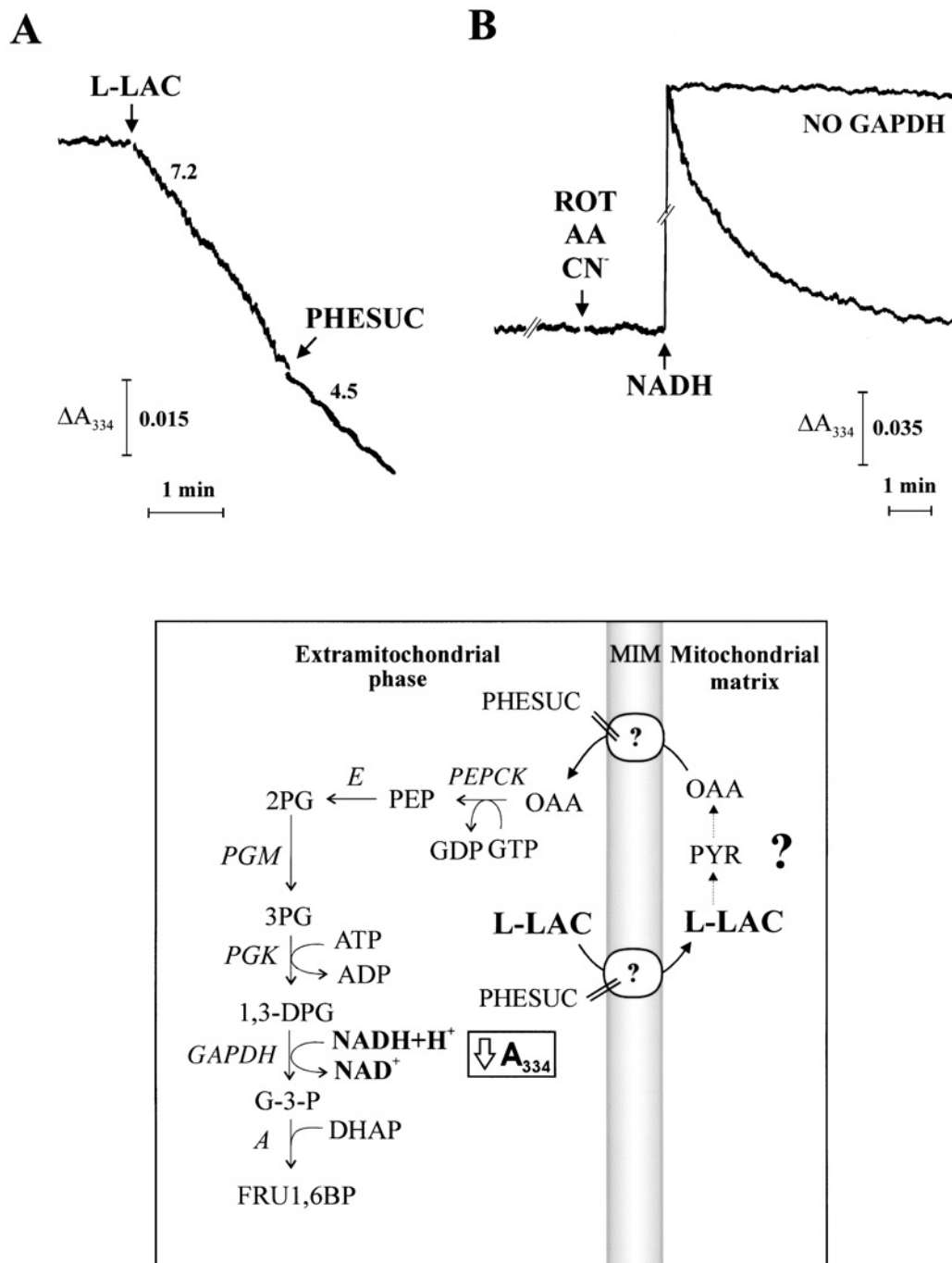
Figure 1   Partial reconstruction of GNG *in vitro*

RLM (1 mg of protein) were suspended at 25 °C in 2 ml of standard medium, consisting of 0.2 M sucrose, 10 mM KCl, 20 mM Hepes/Tris, pH 7.2, 1 mM MgCl$_2$, and G-3-P formation was detected at a wavelength of 334 nm as described in the Experimental section. (**A**) RLM were incubated in the presence of GPRS. At the arrows 10 mM L-lactate (L-LAC) and 10 mM phenylsuccinate (PHESUC) were added. Numbers alongside the curves are rates of G-3-P formation expressed as nmol of NADH oxidized/min per mg of mitochondrial protein. (**B**) RLM plus GPRS were incubated with 10 mM L-lactate (L-LAC) for 5 min; then 2 μg of rotenone (ROT) plus 1.5 μg of antimycin A (AA) plus 1 mM cyanide (CN$^-$) and 0.2 mM NADH were added (arrows). 'NO GAPDH' indicates RLM incubated in the presence of GPRS lacking GAPDH. The Scheme (bottom panel) describes the processes occurring (see text). L-LAC, L-lactate; PEP, phosphoenolpyruvate; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; 1,3-DPG, 1,3-diphosphoglycerate; DHAP, dihydroxyacetone phosphate; FRU1,6BP, fructose 1,6-bisphosphate; PEPCK, phosphoenolpyruvate carboxykinase; E, enolase; PGM, phosphoglycerate mutase; A, aldolase.

by antimycin A (1.5 μg); further addition of ascorbate (5 mM) plus TMPD (0.5 mM) resulted in oxygen consumption which was completely abolished by cyanide (1 mM) (Figure 4A). Both State 4 and State 3 of L-lactate-induced respiration were measured, in the absence or presence of 0.1 mM ADP (Figure 4B). The

respiratory index and P/O value with L-lactate were 2.6 and 3.0 respectively; in the same experiment, the control respiratory index and P/O value with succinate used as a respiratory substrate (5 mM) were 5.0 and 1.9 respectively (results not shown). In five different experiments the P/O values measured for both
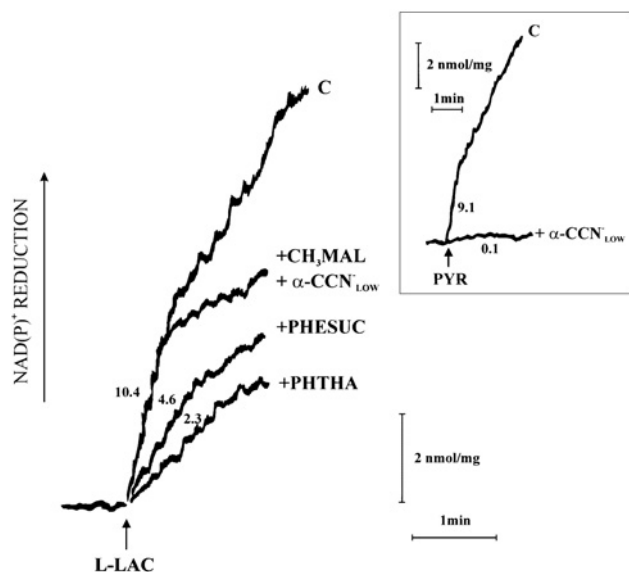
Figure 2   Fluorimetric investigation of the redox state of the mitochondrial pyridine nucleotides caused by externally added L-lactate

RLM (1 mg protein), incubated at 25 °C in 2 ml of standard medium, were treated with 1.25 μM FCCP, used to oxidize intramitochondrial NAD(P)H, and then 2 μg of rotenone (ROT, see Experimental section). Reduction of pyridine nucleotides was followed fluorimetrically (excitation wavelength = 334 nm; emission wavelength = 456 nm) as a function of time. At the arrows, either 5 mM L-lactate (L-LAC) or 0.5 mM pyruvate (PYR; inset) were added to the mitochondrial suspension. Where indicated the following inhibitors were present (added 1 min before the substrate): 10 mM phenylsuccinate (PHESUC), 25 μM α-cyano-4-hydroxycinnamate (α-CCN$^-_{LOW}$), 1 mM phthalonate (PHTHA), 10 mM methylaspartate (CH$_3$MAL). The control curve is labelled 'C'. 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.

L-lactate (5 mM) and the substrate pair malate plus glutamate (5 mM each), were 2.95 ± 0.15 and 3.0 ± 0.1 respectively, and were not statistically different as judged by the Student's t test (results not shown).

L-Lactate (5 mM) proved to increase the mitochondrial membrane potential ($\Delta\Psi$) when added to RLM (Figure 4C), as demonstrated by a decrease in the absorbance of safranine; as a control, the respiratory substrate pair, i.e. malate plus glutamate (both 5 mM), was used to generate mitochondrial $\Delta\Psi$ under the same experimental conditions (Figure 4C, see inset). In both cases, $\Delta\Psi$ generation was inhibited by the electron flow inhibitors; the uncoupler FCCP (1.25 μM) collapsed $\Delta\Psi$ when added to mitochondria after either L-lactate, or malate plus glutamate.

The ability of externally added L-lactate to cause changes in the H$^+$ concentration outside mitochondria was checked both in the absence and the presence of electron flow inhibitors. Externally added L-lactate (5 mM) was found to cause proton release from mitochondria (Figure 5A) in a rotenone-sensitive manner, whereas D-lactate (5 mM) and then the substrate pair ascorbate (5 mM) plus TMPD (0.5 mM) added to RLM after L-lactate, caused rotenone-insensitive proton release inhibited by antimycin A and cyanide respectively, as in [11]. Proton uptake by RLM due to FCCP (1.25 μM) addition was found (Figure 5A, inset).

Following the addition of either 5 mM L-lactate or 0.5 mM pyruvate to mitochondria, previously treated with rotenone, antimycin A and cyanide, used to prevent mitochondrial metabolism, proton uptake by RLM was found (Figure 5B). The initial rate of H$^+$ uptake by mitochondria was inhibited by both 10 mM phenylsuccinate and by 1 mM α-CCN$^-$, but was insensitive to 25 μM α-CCN$^-$. In the same experiment, the proton uptake due to addi-
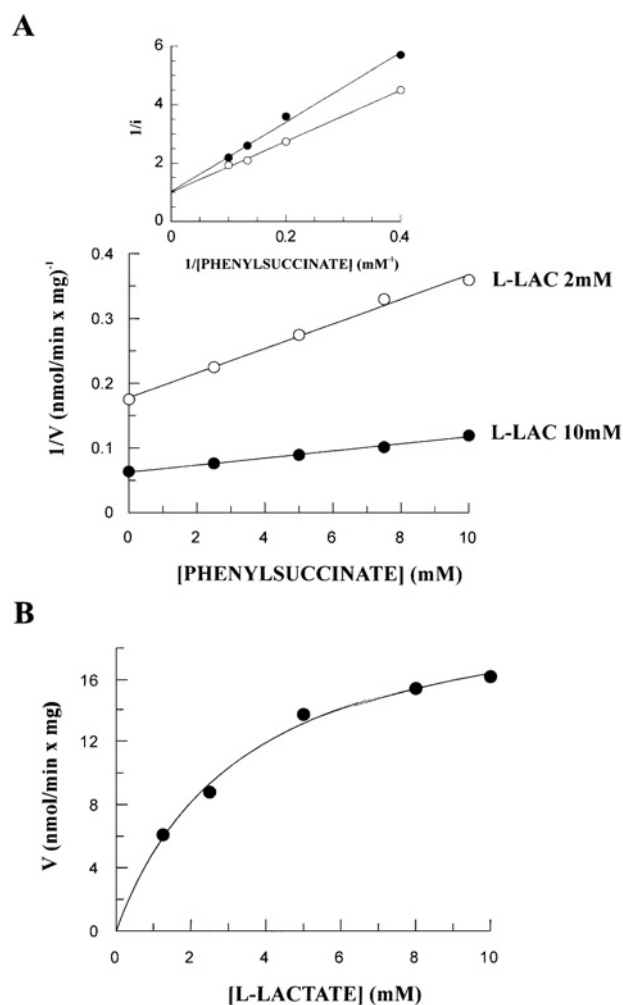

Figure 3   Phenylsuccinate inhibition and saturation characteristics of L-lactate uptake

(**A**) Dixon plot of the inhibition by phenylsuccinate of the rate of reduction of intramitochondrial pyridine nucleotide due to externally added L-lactate. RLM (1 mg of protein) were incubated at 25 °C in 2 ml of standard medium. The rate of reduction of intramitochondrial pyridine nucleotides was measured as in Figure 2, using 2 mM ($\bigcirc$) and 10 mM ($\bullet$) L-lactate (L-LAC) either in the absence or presence of phenylsuccinate at the indicated concentrations. The rate (V), measured as the tangent to the initial part of the progress curve, is expressed in nmol of NADH oxidized/min per mg of mitochondrial protein. The inset is a replot of the data where $i = 1 - V_i/V_o$; V_i and V_o are the rates of L-lactate uptake in the presence or in the absence of the inhibitor. (**B**) Dependence of the rate of reduction of mitochondrial pyridine nucleotides on increasing L-lactate concentrations. L-lactate was added to the mitochondrial suspension (1 mg of protein) at the indicated concentrations with the experiment carried out as described in Figure 2. The rate (V) is expressed as nmol of NAD(P)$^+$ reduced/min per mg of mitochondrial protein.

tion of 0.5 mM pyruvate to the RLM was completely prevented by 25 μM α-CCN$^-$, but was insensitive to 10 mM phenylsuccinate (Figure 5B, inset).

The data reported above indicate that L-lactate can enter mitochondria in a carrier-mediated manner, as shown by the results obtained with inhibitors, and that externally added L-lactate causes proton uptake as expected in the case of a proton-compensated symport. In order to confirm this last conclusion, the fluorescence increase due to addition of L-lactate (5 mM) to mitochondria was monitored in the presence of certain ionophores under experimental conditions designed to affect selectively either ΔpH or $\Delta\Psi$ (Figure 6A). A decrease in the initial rate of intramitochondrial NAD(P)$^+$ reduction was found to occur in the
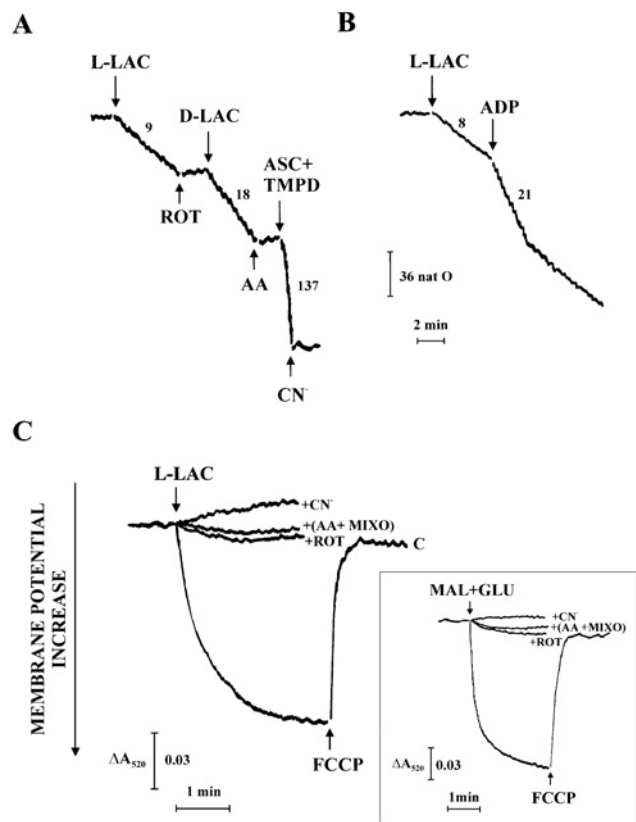
Figure 4   Oxygen uptake and changes in mitochondrial ΔΨ accompanying L-lactate addition to RLM

(**A**, **B**) RLM (1 mg protein) were suspended at 25 °C in 1.5 ml of a medium consisting of 210 mM mannitol, 70 mM sucrose, 3 mM MgCl₂, 0.1 mM EDTA, 5 mM KH₂PO₄/K₂HPO₄, 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: 5 mM L-lactate (L-LAC), 2 μg of rotenone (ROT), 5 mM D-lactate (D-LAC), 1.5 μg of antimycin A (AA), 5 mM ascorbate (ASC) plus 0.5 mM TMPD, 1 mM potassium cyanide (CN⁻), and 0.1 mM ADP. Numbers along the curves are rates of oxygen uptake expressed as ng-atoms of O/min per mg of mitochondrial protein. (**C**) RLM (1 mg protein) were suspended at 25 °C in 2 ml of RLM isolation medium containing safranine (9.6 μM) used as ΔΨ probe. The safranine response was monitored as described in the Experimental section. The following additions were made: 5 mM L-lactate (L-LAC), or malate plus glutamate (MAL + GLU, each 5 mM; inset) and 1.25 μM FCCP. Where indicated, 2 μg of rotenone (ROT) or 1.5 μg of antimycin A (AA) plus 0.5 nmol MIXO or 1 mM potassium cyanide (CN⁻) were added 2 min before the substrate.

presence of nigericin or valinomycin (in the absence of K⁺ ions), which cause a decrease in mitochondrial ΔpH; on the other hand, an increase in the rate was found when pyridine reduction was monitored in the presence of valinomycin plus KCl, which causes an increase in mitochondrial ΔpH by collapsing ΔΨ [see 18]. Consistently, we found that RLM suspended in 0.1 M ammonium L-lactate show spontaneous swelling due to L-lactate uptake. Moreover, the swelling rate and extent in ammonium L-lactate was higher than that found in ammonium D-lactate (0.1 M), indicating that lactate uptake is stereospecific (Figure 6B). As a control, swelling in sucrose, ammonium phosphate and ammonium pyruvate were also monitored (Figure 6B, inset).

L-Lactate-dependent appearance of pyruvate and OAA outside mitochondria

To check whether addition of L-lactate can cause efflux from mitochondria of metabolites newly synthesized in the matrix, the appearance of pyruvate, OAA and malate outside mitochondria
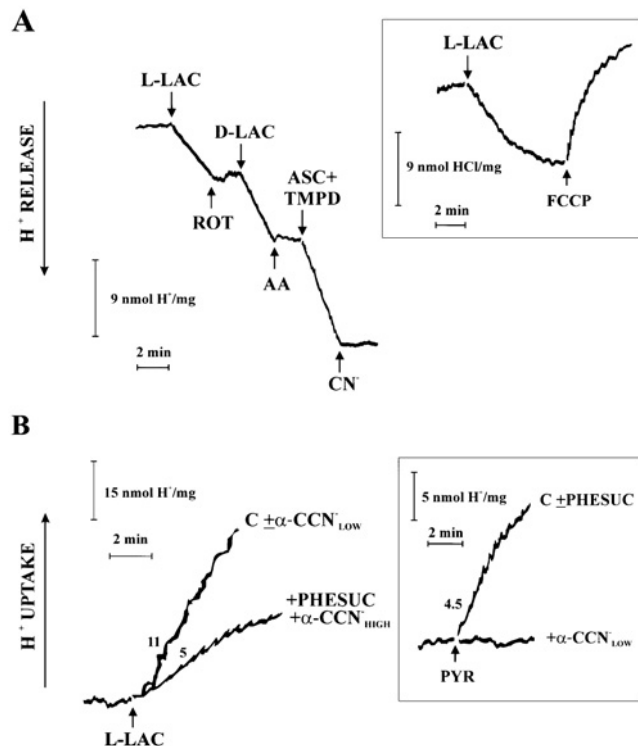

Figure 5   Measurements of proton movement across the mitochondrial membrane due to L-lactate

RLM (1 mg 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). Proton movement was monitored as described in the Experimental section. (**A**) The following additions were made at the arrows: 5 mM L-lactate (L-LAC), 2 μg of rotenone (ROT), 5 mM D-lactate (D-LAC), 1.5 μg of antimycin A (AA), 5 mM ascorbate (ASC) plus 0.5 mM TMPD, 1 mM potassium cyanide (CN⁻), and 1.25 μM FCCP (**A**, inset). In (**B**) RLM were incubated for 3 min with rotenone (2 μg) plus antimycin A (1.5 μg) plus potassium cyanide (1 mM) and then 5 mM L-lactate (L-LAC) or pyruvate (PYR, 0.5 mM; inset) were added. Where indicated either 1 mM or 25 μM α-cyano-4-hydroxycinnamate (α-CCN⁻$_{HIGH}$ or α-CCN⁻$_{LOW}$ respectively) or 10 mM phenylsuccinate (PHESUC) were added 1 min before the substrate. The numbers give the rate of H⁺ uptake, measured as tangents to the initial part of the progress curves and expressed as nmol of H⁺ taken up/min per mg of mitochondrial protein.

was measured photometrically (Figure 7). The pyruvate, OAA and malate concentrations outside RLM were negligible, since no change in A_{334} was found in the presence of PDS (NADH + L-LDH), ODS (NADH + MDH) and MDS (NADP⁺ + M.E.) respectively. As a result of addition of 5 mM L-lactate, pyruvate (Figure 7A) and OAA (Figure 7B) were found outside mitochondria, as shown by the decrease of NADH absorbance. In contrast with the situation concerning the D-isomer [11], addition of L-lactate caused no appearance of malate (Figure 7C), as shown by lack of change in NADPH absorbance. Interestingly, the metal complexing agent bathophenanthroline (0.1 mM) was found to inhibit the appearance of AA, but not that of pyruvate, whereas α-CCN⁻ inhibited both the effluxes.

RLM were incubated with L-LDH or MDH plus NADPH (0.2 mM) followed by addition of 5 mM L-lactate (insets of Figures 7A and 7B). After TX-100 (0.5%) was added to RLM, a rapid decrease of absorbance due to solubilization of the mitochondria occurred; the rate of L-lactate-dependent pyruvate (Figure 7A, inset) and of OAA (Figure 7B, inset) production was found to increase, thus showing that L-lactate transport across the mitochondrial membrane in exchange with these metabolites is the rate-limiting step of the measured rate of absorbance change.
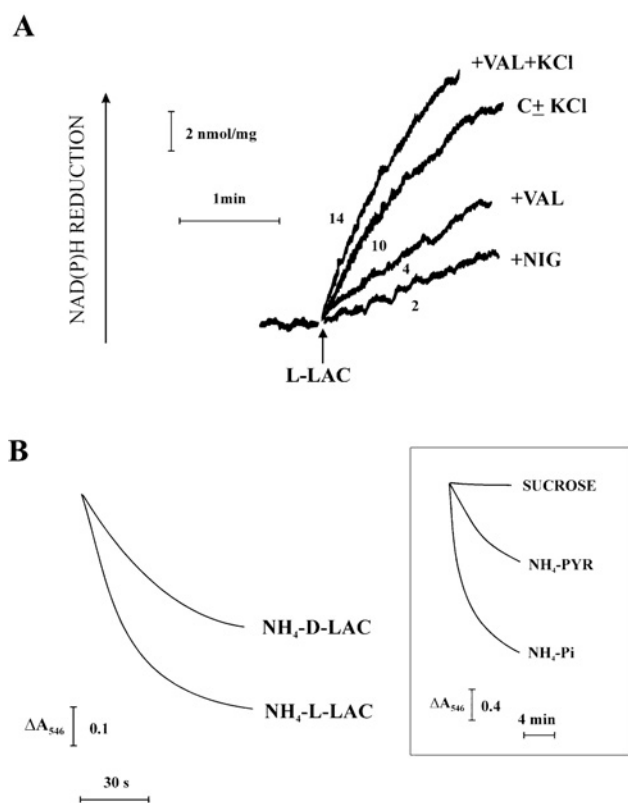
Figure 6 The effect of certain ionophores on the rate of reduction of mitochondrial NAD(P)$^+$ due to L-lactate (A) and on mitochondrial swelling in ammonium lactate solutions (B, C)

(**A**) RLM (1 mg of protein) were incubated for 3–5 min at 25 °C in 2 ml of a medium consisting of 0.22 M sucrose, 20 mM Hepes/Tris, pH 7.2, 1 mM MgCl$_2$. Where indicated, 25 mM KCl was also present and the sucrose concentration was 0.17 M. Pyridine coenzyme reduction was followed fluorimetrically as described in Figure 2. At the arrows, 5 mM L-lactate (L-LAC) was added in the absence (C) or presence of 0.5 μg of valinomycin (+VAL), or 0.5 nmol nigericin (+NIG), added 3 min before the substrate. 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. (**B**) 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 (inset), 0.1 M ammonium L-lactate (NH$_4$-L-LAC), 0.1 M ammonium D-lactate (NH$_4$-D-LAC) and mitochondrial swelling were monitored as described in the Experimental section.
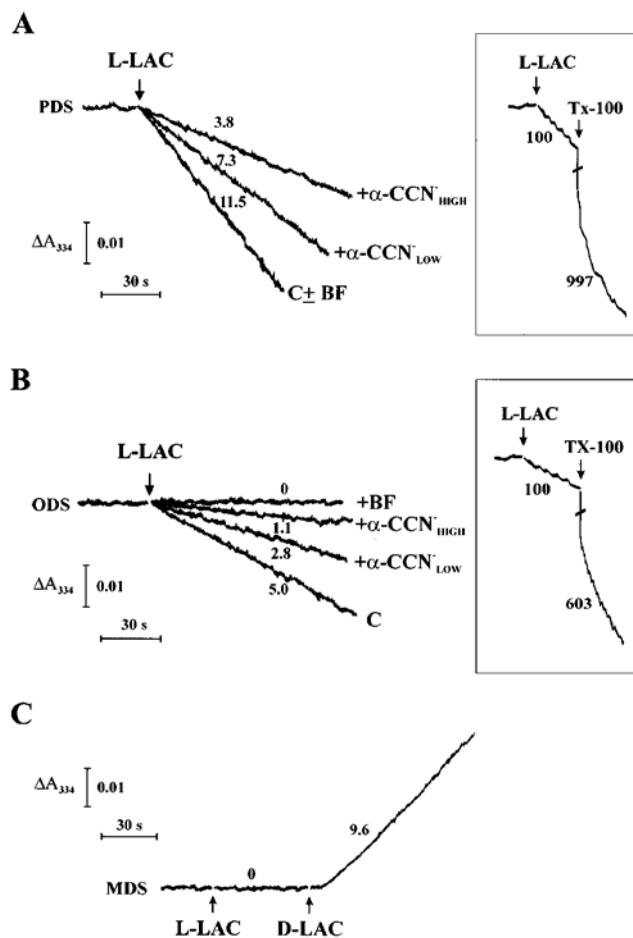

Figure 7 Export of pyruvate and OAA induced by the addition of L-lactate to RLM

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 L-lactate dehydrogenase); (**B**) the ODS (0.2 mM NADH plus 2 units malate dehydrogenase); (**C**) the MDS (0.25 mM NADP$^+$ plus 0.2 unit ME). At the arrow 5 mM L-lactate (L-LAC) was added in the absence (C) or presence of the following inhibitors (added 1 min before the substrate): 0.1 mM bathophenathroline (BF) or either 1 mM or 25 μM α-cyano-4-hydroxycinnamate (α-CCN$^-_{HIGH}$ or α-CCN$^-_{LOW}$ respectively). In (**C**) 5 mM D-lactate (D-LAC) was added after L-lactate at the time indicated by the arrow. The numbers give the rate of A_{334} changes, 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. In the insets 10 mM L-lactate (L-LAC) and 0.5 % TX-100, were added to RLM incubated with 0.2 mM NADPH plus either 1 unit L-LDH (inset **A**) or 2 units malate dehydrogenase (inset **B**). Numbers along the curves are the rates of absorbance change expressed as nmol/min per mg protein.

In the light of the transport paradigm according to which mitochondria first provide net carbon uptake and then allow for efflux of newly synthesized compounds (see [25]), a possible explanation of these findings is that L-lactate enters RLM via an L-lactate/H$^+$ symporter; pyruvate and OAA are synthesized in the matrix because of the presence of the mitochondrial L-LDH and the other GNG mitochondrial enzymes. Both pyruvate and OAA can, in turn, move to the extramitochondrial phase in exchange with further L-lactate in a carrier-mediated manner.

Consistently phenylsuccinate was found to inhibit L-lactate/OAA antiport (Figure 8A) in a competitive manner, and to inhibit L-lactate/pyruvate antiport in a non-competitive manner (Figure 8B). In both cases application of control strength criteria (see above) showed that phenylsuccinate-sensitive L-lactate transport controls the rate of the measured process, i.e. the rate of appearance of pyruvate and of OAA outside RLM. The data from Figures 8(A) and 8(B), were plotted as 1/i against 1/[phenylsuccinate], as in Figure 3(A). That the y intercepts were equal to unity, further excludes the possibility both of spontaneous OAA efflux

from mitochondria and of pyruvate efflux occurring in a manner not sensitive to phenylsuccinate.

The dependence of the rate of appearance of pyruvate and OAA, in the extramitochondrial phase, on the concentration of L-lactate externally added to RLM was determined and the results analysed by means of a double reciprocal plot (Figures 9A and 9B respectively). The reactions revealed hyperbolic characteristics. K_m and V_{max} values were equal to 4.2 mM and 20 nmol/min per mg (L-lactate/pyruvate), and to 5 mM and 10 nmol/min per mg (L-lactate/OAA). In four experiments, carried out with different mitochondrial preparations, the V_{max} values of the L-lactate/pyruvate and L-lactate/OAA exchanges were found to be different as judged by a statistical analysis carried out by using the Student's t test. The mean V_{max} values were 19 ± 1.8 and 10 ± 0.8 respectively; the
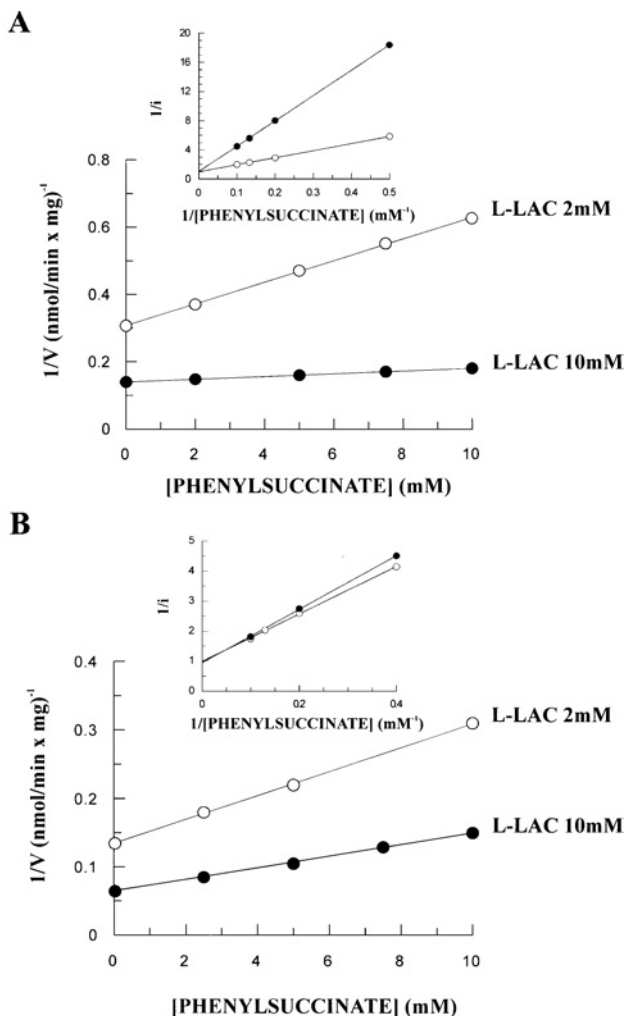
A

B

Figure 8   Dixon plots of the inhibition by phenylsuccinate of the rate of L-lactate/OAA and L-lactate/ pyruvate exchange

RLM (1 mg of protein) were incubated at 25 °C in 2 ml of standard medium. The OAA or pyruvate efflux rate was measured as in Figure 7, by using 2 mM (○) and 10 mM (●) L-lactate (both in **A** and in **B** respectively) either in the absence or presence of phenylsuccinate at the indicated concentrations. The rate (V), measured as the tangent to the initial part of the progress curve, is expressed in nmol of NADH oxidized/min per mg of mitochondrial protein. The insets are replots of the data where $i = 1 - V_i/V_o$; V_i and V_o are the rates of L-lactate/OAA exchange (**A**, inset) or L-lactate/pyruvate exchange (**B**, inset) in the presence or in the absence of the inhibitor.

mean K_m values were 4.1 ± 0.2 and 5.1 ± 0.2 mM respectively. The L-lactate symporter ($V_{max} = 22.5 \pm 1.5$ nmol/min per mg protein and $K_m = 3.3 \pm 0.3$ mM) was clearly different from the L-lactate/OAA antiporter, but on the basis of the measured kinetic parameters it could not be distinguished from the L-lactate/ pyruvate antiporter.

Inhibition and pH profiles of L-lactate transport

The proton-compensated L- and D-lactate uptake, the L- and D-lactate-dependent metabolite effluxes, and the monocarboxylate-carrier-mediated transport were compared with respect to their sensitivity to a variety of compounds including bathophenanthroline, α-CCN$^-$ (either 25 μM or 1 mM), methyl-DL-alanine, DL-β-amino-n-butyrate, phenylsuccinate, methylmaleate, methylaspartate, methylglutamate and phthalonate. The L- or
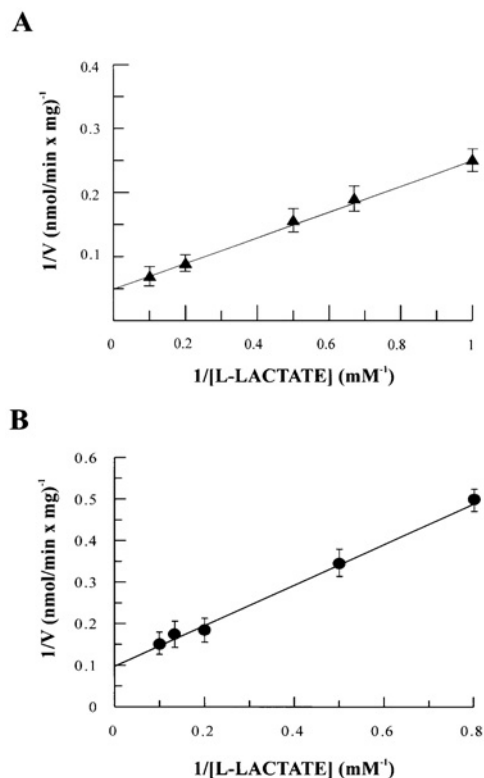

A

B

Figure 9   The dependence of the L-lactate/pyruvate and L-lactate/OAA exchange rates on increasing L-lactate concentrations

RLM (1 mg of protein) were suspended in 2 ml of standard medium under the same experimental conditions reported in Figure 7. L-lactate was added to the mitochondrial suspension at the indicated concentrations. L-lactate/pyruvate (**A**) and L-lactate/OAA (**B**) exchange rates were measured as tangents to the initial part of the progress curves and expressed in nmol of NADH oxidized/min per mg of mitochondrial protein.

D-lactate and pyruvate concentrations were 2 mM and 0.2 mM respectively, with the inhibitor concentrations varying between 10 and 1 mM, except for bathophenanthroline which was 0.1 mM (Figure 10). Of particular interest is that sensitivity to bathophenanthroline showed the L-lactate/OAA antiporter to be different from the L-lactate/pyruvate antiporter, L-lactate/H$^+$ symporter and D-lactate/oxoacid antiporter, whereas on the basis of its sensitivity to methylmaleate and 1 mM α-CCN$^-$, the L-lactate/pyruvate antiporter can be distinguished from the L- and D-lactate/H$^+$ symporters, monocarboxylate carrier and D-lactate/oxoacid antiporter. Finally, L-lactate/H$^+$ symporter can be distinguished from the two novel L-lactate antiporters, the D-lactate/H$^+$ symporter and the pyruvate carrier by virtue of the N-methylglutamate sensitivity.

L-lactate/H$^+$ symport, monitored fluorimetrically, as reported in Figure 2, and L-lactate-dependent pyruvate and OAA efflux measured photometrically, as reported in Figure 7, were also compared on the basis of their pH profiles (Figure 11). In three experiments, carried out with different mitochondrial preparations, the pH profiles were found to differ from each other. The rates of transport were increased by decreasing pH for both L-lactate/H$^+$ symport (Figure 11A) and L-lactate/OAA antiport (Figure 11B), whereas the rate of L-lactate/pyruvate antiport, which is minimum at pH 6.5, increased with increasing pH (Figure 11C). In each experiment, 5 mM L-lactate was added to RLM to start the reaction. When determining the pH profiles we confirmed that the activity of the substrate-detecting systems was higher than the rate of metabolite efflux; moreover the application of control strength
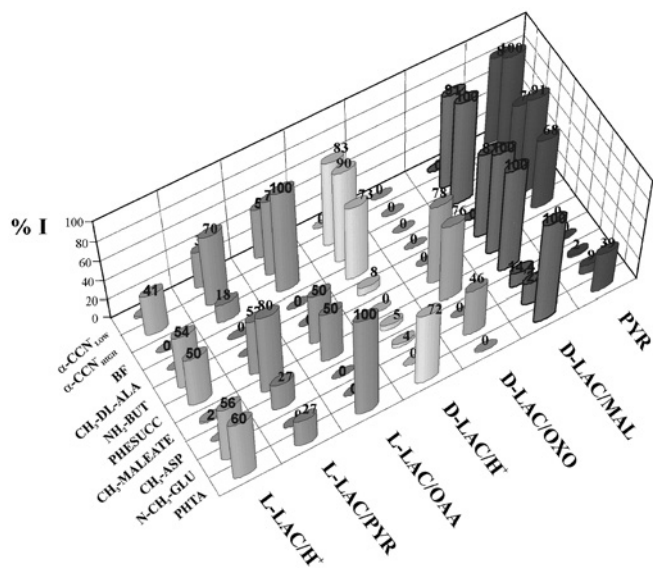
Figure 10 The sensitivity of the L-lactate/pyruvate and L-lactate/OAA antiports and of the L-lactate/H⁺ symport to a variety of compounds

The transport processes were measured, as described in the Experimental section and in Figures 2 and 7, by using 2 mM L-lactate, 2 mM D-lactate and 0.2 mM pyruvate either in the absence or presence of the following compounds (added to RLM 1 min before the substrate): 0.1 mM bathophenanthroline (BF), α-cyano-4-hydroxycinnamate (α-CCN⁻$_{HIGH}$ or α-CCN⁻$_{LOW}$, 1 mM or 25 μM respectively), 1 mM phthalonate (PHTA). Methyl-DL-alanine (CH$_3$-DL-ALA), DL-β-amino-n-butyrate (NH$_2$-BUT), phenylsuccinate (PHESUC), methylmaleate (CH$_3$-MALEATE), methylaspartate (CH$_3$-ASP) and N-methylglutamate (N-CH$_3$-GLU) were used at concentration equal to 10 and 1 mM in the case of L- or D-lactate and pyruvate respectively.

criterion showed that in the pH range investigated the rate of absorbance change mirrored the rate of transport.

DISCUSSION

The occurrence of L-lactate metabolism in mitochondria [8,10,27,28] poses previously unaddressed questions as to whether and how L-lactate oxidation and export of GNG metabolites from mitochondria can contribute to GNG. Consequently, we investigate the capability of RLM incubated with L-lactate in the presence of an enzyme/cofactors cocktail which allows for partial reconstruction of GNG *in vitro* to produce G-3-P. Appearance of G-3-P outside mitochondria was established. We showed that this occurred because of the capability of RLM to take up externally added L-lactate, metabolize it in the matrix and export the newly synthesized OAA out of the mitochondria (Scheme 1). This is made possible by the existence of an L-lactate/H⁺ symporter, the L-LDH located in the matrix, and an L-lactate/OAA antiporter. An L-lactate/pyruvate antiporter may participate by removal of reducing equivalents from the cytosol via the L-lactate/pyruvate shuttle already reported for heart [12].

The role of L-lactate metabolism and transport by RLM will be discussed separately.

L-LDH

This paper confirms that RLM contain their own NAD-dependent L-LDH, localized in the matrix space. We show that intramitochondrial pyridine nucleotide reduction, oxygen uptake, membrane potential generation, proton release and ATP synthesis with P/O ratio = 3 all occur as a result of L-lactate uptake. None of these events could occur unless L-lactate enters mitochondria
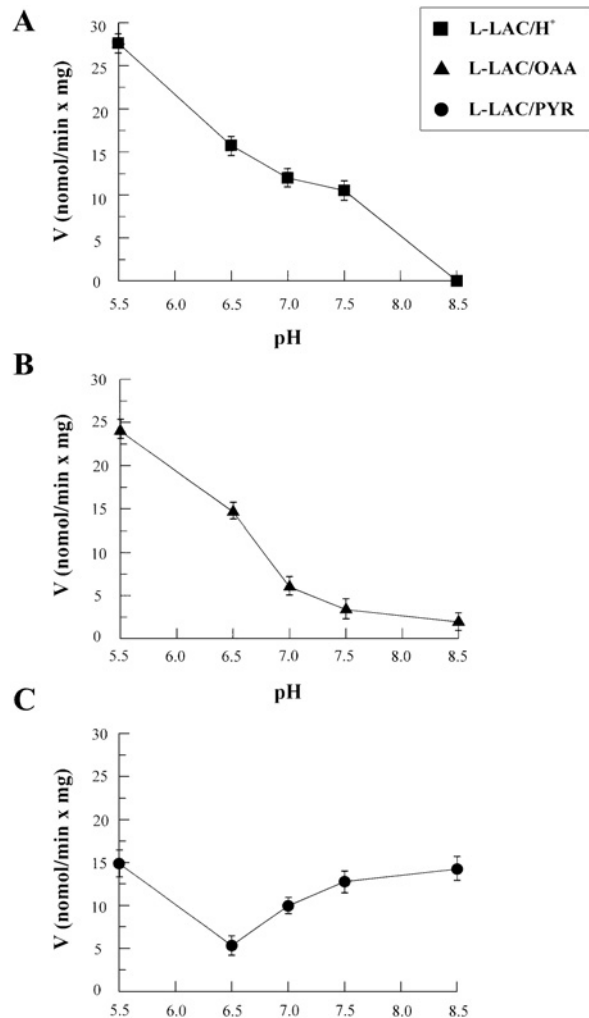

Figure 11   pH profiles of L-lactate transport in RLM

RLM (1 mg of protein) were incubated at 25 °C in 2 ml of standard medium whose pH was adjusted to the indicated values with either Tris or HCl. Experimental conditions were as in Figure 2 for L-lactate/H⁺ symport (**A**), and in Figure 7, for L-lactate/pyruvate (**B**) and L-lactate/OAA (**C**) antiporters. L-lactate concentration was 5 mM. The rates were measured as tangents to the initial part of the progress curves and expressed in nmol of NADH oxidized/min per mg protein.

where NAD⁺ is located, and in addition we provide direct evidence that external L-LDH found in our mitochondrial preparation cannot affect our measurements (see Experimental section). The occurrence of an NAD-linked L-LDH located in the intermembrane space has been also proposed [5,8], and it could be argued that this could lead to an over-estimation of intramitochondrial pyruvate formation in our experiments. This possibility can be ruled out since no NAD⁺ was present outside mitochondria as judged via HPLC carried out as in [29]. In addition, we carried out a control experiment in which we measured the kinetics of the three L-lactate transport processes in mitoplasts; the K_m values were found not to differ from those calculated in RLM, and the V_{max} values were all increased by the percentage expected as a result of removal of inert protein.

The efflux of both pyruvate and OAA could remove the major theoretical argument raised (see [10]) against the occurrence of L-LDH in mitochondria. It was argued that since the NAD⁺/NADH ratio is approx. 1000 in cytosol and less than 10 in the matrix [10], this should prevent any mitochondrial L-lactate
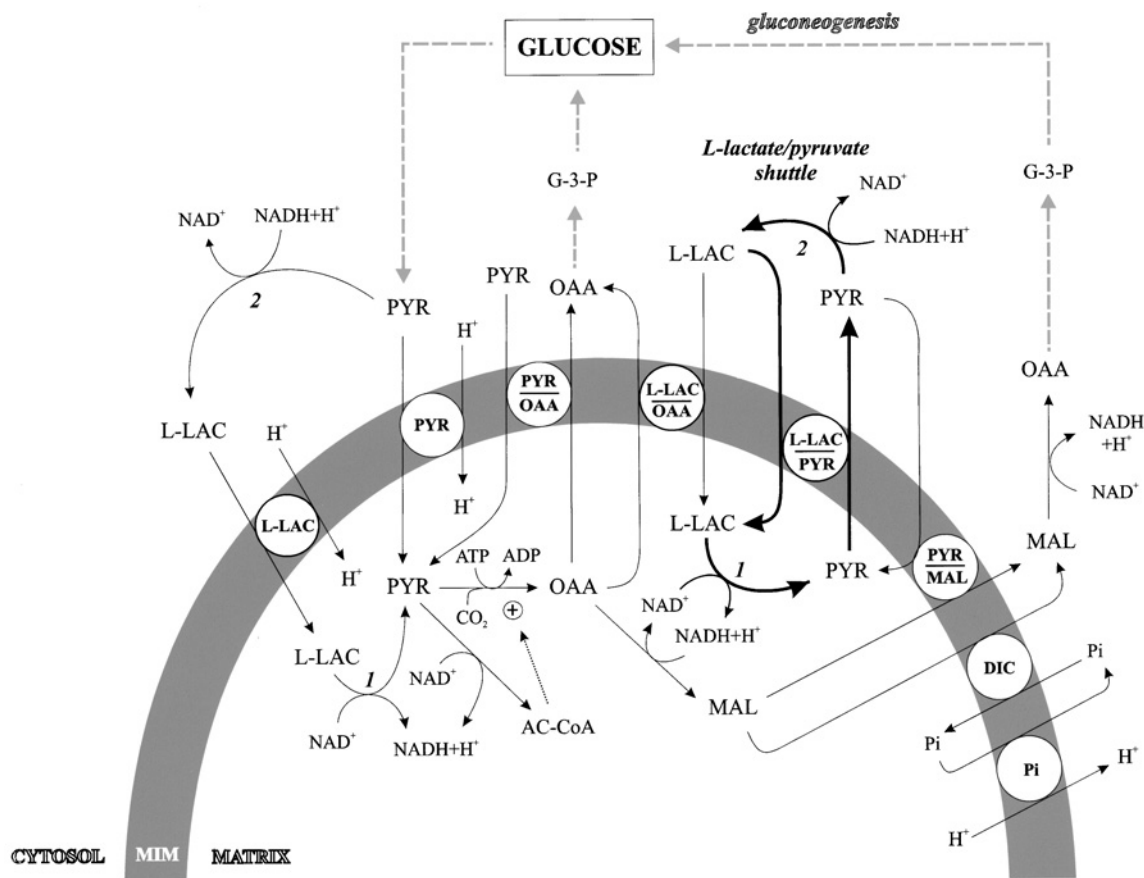
Scheme 1   L-**Lactate transport and metabolism in RLM**

For explanation see the text. Abbreviations: (1) mitochondrial L-lactate dehydrogenase; (2) cytosolic L-LDH; L-LAC, L-lactate; MAL, malate; PYR, pyruvate; OAA, oxaloacetate; G-3-P, glyceraldehyde-3-phosphate; MIM, mitochondrial inner membrane. Carriers: DIC, dicarboxylate carrier; P_i, phosphate carrier; PYR/MAL, pyruvate/malate antiporter; PYR/OAA, pyruvate/OAA antiporter; PYR, monocarboxylate transporter; L-LAC/PYR, L-LAC/pyruvate antiporter; L-LAC/OAA, L-LAC/OAA antiporter; L-LAC, L-LAC/H$^+$ symporter.

metabolism since the lactate/pyruvate ratio is approx. 100 times higher in the mitochondria than in the cytoplasm; accordingly, L-lactate oxidation is basically much more likely in the cytoplasm than in the mitochondria. However, the removal of the oxidation product by carrier-mediated transport and mitochondrial metabolism overcomes this thermodynamic difficulty.

The L-lactate symporter and the L-lactate antiporters

In order to study L-lactate uptake by RLM, we first resorted to isotopic techniques, by checking the capability of labelled lactate to enter mitochondria using the stop inhibitor method [25]. No significant accumulation of radioactivity was found, but this is easily explained by the ability of compounds newly synthesized in the matrix to efflux from the mitochondria. Thus we resorted to spectroscopic methods to monitor mitochondrial reactions and traffic of the newly synthesized substrates across the mitochondrial membrane. Ample evidence was obtained (see Figures 3,7,8) that the measured changes of both fluorescence and absorbance mirrored the rates of transport processes.

The existence of a carrier that mediates L-lactate/H$^+$ symport in RLM is shown by the hyperbolic kinetics of the transport, as measured fluorimetrically, and by the inhibition studies. Since L-lactate uptake by RLM can also occur in exchange for pyruvate and OAA, it could be argued that the rate of NAD(P)H fluore-

scence change reflects the activity of both L-lactate symporter and antiporters. However, the failure of externally added methylmaleate and 25 μM α-CCN$^-$ (both of them shown to inhibit the antiports) to inhibit the initial rate of NAD(P)H reduction establishes that what is being measured is uptake solely due to the symporter. That mitochondria can import L-lactate in a proton compensated manner with net carbon uptake was also shown by measurements of swelling in isotonic ammonium L-lactate solutions. This conclusion was also strongly supported by experiments with specific ionophores which provided a correlation between ΔpH and swelling rate (results not shown), and between ΔpH and L-lactate uptake as measured by NAD(P)H fluorescence increase. Finally, proton uptake by mitochondria accompanying L-lactate import was shown under conditions where L-lactate oxidation was 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 [18].

The saturation kinetics observed for pyruvate and OAA export under conditions that reflect the transport step of the measured process, show that the effluxes occur in a carrier-mediated manner (Figure 9). Since both free diffusion and use of the monocarboxylate carrier have been ruled out experimentally (see above), we conclude that three L-lactate carriers exist in RLM, and that they are distinguished by the differences found in the V_{max}

values and, more importantly, by the inhibition and pH profiles (Figures 10,11). Moreover, a comparison between the inhibition profiles of the novel L-lactate carriers and other carriers, show that they can be also distinguished from others already shown in RLM, including the dicarboxylate, the tricarboxylate, and the oxo-glutarate carriers. Moreover, we can exclude the possibility that the OAA efflux is a result of the combined action of the L-lactate/pyruvate and of the pyruvate/OAA [21] and pyruvate/malate [14] antiporters, since the V_{max} of the pyruvate/OAA and pyruvate/malate carriers in RLM are very low, and are not consistent with those measured in this study.

The existence of different carriers for the same metabolite, one of them producing net carbon uptake is not unique, and is consistent with the transport paradigm [25] according to which net uptake occurs via a uni/symporter, after which newly-synthesized compounds can be exported by antiport.

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

The role of mitochondrial L-lactate transport and metabolism

To date, L-lactate metabolism in liver has been considered solely in the light of the cytosolic L-LDH that starts GNG from L-lactate. Our results, which show the occurrence of mitochondrial metabolism of L-lactate and, more importantly, the ability of L-lactate to export OAA out of the mitochondria, give new insights into hepatic GNG. The additional participation of mitochondria in hepatic GNG, as proposed in the light of the results reported, is shown in Scheme 1. Externally added L-lactate can enter mitochondria and cause the efflux of pyruvate and OAA in a carrier-mediated transport. Consistently, we show the existence of three carriers for L-lactate transport in mitochondria. Two of them, the L-lactate symporter and the L-lactate/OAA antiporter, join those reported previously, which participate in GNG including the monocarboxylate, the pyruvate/OAA, the pyruvate/malate, the D-lactate symporter, the D-lactate/oxoacid, D-lactate/malate [11], and the dicarboxylate carrier. Such abundance is not surprising since it accounts for the differences in GNG under a variety of physiological conditions.

How much OAA exported to the cytosol in exchange for L-lactate contributes to GNG, in addition to that exported as aspartate and/or malate, can be only a matter of speculation. By assuming that the rate of glucose formation is about 0.5–1 μmol/min per g cell protein [30,31], and by considering that the rate of OAA efflux at 10 mM L-lactate is 7 nmol/min per mg mitochondrial protein and that mitochondrial protein constitutes 20% of the total cell protein, we can calculate that at least 0.14 μmol of the newly synthesized glucose can derive from the OAA produced in mitochondria from imported L-lactate. That is, it can make a 20–40% contribution to glucose production. One could argue that the cytosolic L-LDH is far more active than the L-lactate symporter, thus preventing L-lactate uptake from occurring. However, the occurrences of matrix L-LDH and of L-lactate traffic across the mitochondrial matrix are matters of fact, and strongly suggest that L-lactate does enter mitochondria. Another argument against mitochondrial L-lactate GNG, as proposed here, could be the need for export of reducing equivalents from mitochondria [4]. However, assuming that the rate of L-lactate oxidation in the cytosol is higher than that of the symport, and that the GNG rate depends on the pyruvate uptake [32], we can conclude that cytosol NADH production via L-LDH provides a reducing equivalent level high enough to allow for GNG from OAA exported from mitochondria to occur. Since a high cytosol NADH/NAD$^+$

ratio could result in inhibition of GNG [33], we propose that the additional L-lactate/pyruvate shuttle shown here, as in [12], could work as a safety valve. Interestingly it was reported that at low lactate concentration the rate of GNG is determined by pyruvate transport, whereas at 10 mM L-lactate this does not occur, which argues for both excess capacity and a significant alternative pathway [34]; this alternative could well be the lactate/mitochondria pathway shown in this study. In this regard, we also suggest that the residual GNG which is known to occur in the presence of transaminase inhibitors [4,35–37] could be due to L-lactate mitochondrial transport and metabolism.

In summary, we suggest that the additional pathway for hepatic GNG described here should join the 'classic' GNG pathways reported previously [4].

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