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STUDIES ON THE ACTIVE TRANSFER OF REDUCING EQUIVALENTS INTO MITOCHONDRIA VIA THE MALATE-ASPARTATE SHUTTLE

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SUMMARY

1. The effects of mitochondrial energy states on the extramitochondrial NADH/NAD ratio via a reconstituted malate-aspartate shuttle have been investigated.

2. The transfer of reducing equivalents into isolated mitochondria is stimulated by ATP and by electron transport. The effect of ATP is inhibited by oligomycin. The effect of electron transport is inhibited by uncouplers.

3. Uncoupling of the mitochondria is required for rapid transfer of reducing equivalents out of the mitochondria.

4. A glutamate-stimulated entry of aspartate into energized mitochondria suggests that the malate-aspartate shuttle is to some extent reversible even in a high energy state of the mitochondria.

5. It is concluded that the malate-aspartate shuttle contributes to the formation of the skewed redox situation across the inner mitochondrial membrane, which has a more reduced inside.

INTRODUCTION

The established impermeability of the mitochondrial membrane to NADH caused a search for mechanisms by which cytoplasmic NADH could be oxidized by mitochondria. A generally accepted mechanism, the so-called malate-aspartate shuttle, was suggested by Borst [1] in 1963. The operation of this shuttle has been confirmed in studies on perfused livers [2] and hearts [3] and isolated liver cells [4]. The shuttle can be reconstituted in vitro and catalyzes an efficient oxidation of added NADH in isolated mitochondria [5–7].

One difficulty in understanding the operation of the malate-aspartate shuttle in vivo is the NADH/NAD ratio which is much lower in the extramitochondrial cytoplasm than in the mitochondria [8]. The difference in ratio (with a factor of as

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much as 100) implies that NADH is taken up against a gradient and that an energy input is needed.

The operation of the shuttle implies uptake of malate and glutamate and expulsion of aspartate and α-ketoglutarate by the mitochondria. Active transport of any of these metabolites across the mitochondrial membrane will represent an energy input in the system. Recently LaNoue et al. [9] have shown that aspartate is actively transported out of mitochondria in exchange for glutamate, probably by an electrogenic mechanism. This transport may represent the main energy input in the system. It has also been found that malate and other carboxylic acids accumulate in mitochondria, probably because of a pH gradient across the mitochondrial membrane (alkaline inside) [10].

In the present paper we have studied the effect of energy state of the mitochondria on a reconstituted system, which permits the detection of movement of reducing equivalents through the mitochondrial membrane in both directions. Mitochondria and other components were added to a preincubated system where a certain NADH/NAD ratio was in equilibrium with malate, oxaloacetate, aspartate, glutamate and α-ketoglutarate via the enzymes malate dehydrogenase and glutamate oxaloacetate transaminase. Depending on the additions made, the extramitochondrial system became more reduced or more oxidized. An active uptake of reducing equivalents was demonstrated in energized mitochondria. Uncoupling of the mitochondria was required before a rapid outward flux of reducing equivalents could be obtained via the malate-aspartate shuttle.

MATERIALS AND METHODS

Materials

Malate dehydrogenase (EC 1.1.1.37), aspartate aminotransferase (glutamate-oxaloacetate transaminase) (EC 2.6.1.1), glutamate dehydrogenase (EC 1.4.1.2), β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), alcohol dehydrogenase (EC 1.1.1.1), lactate dehydrogenase (EC 1.1.1.27), NAD, NADH, ATP, ADP, rotenone, fatty acid-free bovine albumin, and aminooxyacetate were all obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Malic dehydrogenase and glutamate oxaloacetate transaminase to be added to incubations with mitochondria were dialyzed overnight against 10 mM Tris buffer, pH 7.4, to remove ammonium sulphate. (−)Palmitylcarnitine was prepared according to Bremer [11]. Rat liver mitochondria were sedimented from a 10 % liver homogenate at $8000 \times g$ for 10 min after removal of nuclei and cell debris at $800 \times g$ for 5 min. The mitochondria were washed twice with 0.25 M sucrose and finally suspended in 0.15 KCl, usually to a concentration of 20–30 mg protein per ml. The mitochondria showed good respiratory control when tested with a Clark oxygen electrode.

Incubations

Incubations were performed in approximately 30 mM imidazole/HCl buffer, pH 7.4 (added as a 0.15 M solution). Substrates were usually added as 0.1 M solutions. The final volume was made up with 0.15 M KCl. In experiments with palmitylcarnitine, approximately 0.4 % fatty acid-free bovine albumin was added to the incubation mixture to protect the mitochondria from the detergent effect. Substrate concentra-

tions and other additions are given in the legends to tables and figures. In experiments where NADH formation and/or disappearance was measured, an extramitochondrial equilibrium between a certain NADH/NAD ratio and the malate-aspartate shuttle intermediates was established before the addition of mitochondria. This was done by a preincubation of NAD, malate, glutamate, malate dehydrogenase and glutamate oxaloacetate transaminase. When a low NADH/NAD ratio was desired, aspartate and α-ketoglutarate were also included in the preincubation mixture. Blanks were always run where an equal volume of 0.15 KCl was added instead of mitochondrial suspension. Samples were taken from this blank incubation at the same time as from the mitochondrial incubations. No significant change in the NADH/NAD ratio was found in these controls.

The formation and/or disappearance of NADH was measured after stopping the reaction by transferring samples into test tubes in a boiling water bath. After 60–90 s the test tubes were cooled on ice. The precipitated proteins were removed by centrifugation. Tests showed that the NADH was completely stable to this treatment in imidazole buffer at pH 7.4. In all other experiments the incubations were stopped by addition of 1/4 volume of 2 M $HClO_4$. After removal of the precipitated proteins the supernatant was neutralized with M K_2CO_3. Samples of this neutralized extract were used in the different assays.

Assays

All metabolites were measured enzymically, in a spectrophotometer at 340 nm, coupled to the appearance or disappearance of NADH. Malate and β-OH-butyrate were measured with malate dehydrogenase and β-hydroxybutyrate dehydrogenase, respectively, at pH 9 with approximately 0.3 M hydrazine as trapping agent for oxaloacetate and acetoacetate. Oxoglutarate was measured by conversion to glutamate by glutamate dehydrogenase in the presence of excess amounts of NADH. Aspartate was measured by converting it to malate in the presence of α-ketoglutarate, NADH, glutamate, oxaloacetate transaminase and malic dehydrogenase. NADH was assayed by measuring the decrease in optical density at 340 nm after addition of pyruvate and lactic dehydrogenase. NAD was assayed by measuring the increase in optical density at 340 nm after addition of ethanol and alcohol dehydrogenase.

RESULTS

Table I shows how substrates and energy states affect an extramitochondrial $NADH/NAD^+$ ratio in a reconstituted malate-aspartate shuttle system. The only condition found in which the mitochondria caused reduction of extramitochondrial NAD was when rotenone was added to the incubation mixture. Even succinate and palmitylcarnitine, which are known to produce extremely high mitochondrial NADH/NAD ratios in state 4, were unable to cause a reduction of extramitochondrial NAD, although they diminished the rate of oxidation of extramitochondrial NADH.

While rotenone alone led to reduction of extramitochondrial NAD, rotenone plus succinate or palmitylcarnitine paradoxically led to oxidation of extramitochondrial NADH in spite of the extremely high intramitochondrial NADH/NAD ratios produced by rotenone plus palmitylcarnitine [12].

Uncoupling of the mitochondria with dinitrophenol increased the rate of

TABLE I

REVERSIBILITY OF THE MALATE-ASPARTATE SHUTTLE IN RAT LIVER MITOCHONDRIA

Malate, 3.3 mM; glutamate, 3.3 mM; NAD, 1.7 mM; malate dehydrogenase, approximately 90 units; and glutamate oxaloacetate transaminase, approximately 20 units; were preincubated for 20 min in imidazole/HCl buffer (pH 7.4, 25 mM); KCl (approximately 75 mM); and bovine serum albumin, 0.3 %. After additions as shown, rat liver mitochondria (6 mg of protein, Exp. I; and 9 mg of protein, Exp. II) were added, and the incubation continued as shown. During the preincubation period an equilibrium concentration of 106 and 113 mM NADH had been formed. (The same concentration, about 0.1 mM, of asparatate and α-ketoglutarate had also been formed.) The results are given as increase (+) or decrease (−) in this concentration. Total volume was 3 ml and the temperature was 36 °C.

Additions	Δ NADH	
Experiment I	7 min	20 min
none	+ 80	+102
dinitrophenol, 10^{-3} M	−104	− 105
succinate, 3.3 mM	+ 17	+ 41
palmitylcarnitine, 0.1 mM	− 63	− 92
rotenone, 15 μM	+ 15	+ 42
rotenone + succinate	+ 9	+ 26
rotenone + palmitylcarnitine	− 11	− 38
rotenone + ATP, 1.7 mM	− 25	− 44
rotenone + palmitylcarnitine + dinitrophenol	+ 42	+ 67
rotenone + phosphate, 1.7 mM	+ 36	+ 67
rotenone + palmitylcarnitine + phosphate	− 22	− 69
Experiment II		15 min
none		−110
rotenone, 15 μM		+ 68
rotenone − dinitrophenol		+ 125
rotenone + arsenite, 1 mM		+ 30
rotenone + palmitylcarnitine + arsenite		+ 25
rotenone + palmitylcarnitine + arsenite + dinitrophenol		+130
rotenone + arsenite + ATP		− 54

oxidation of extramitochondrial NADH. However, if rotenone was also added, uncoupling stimulated the reduction of extramitochondrial NAD. Thus, uncoupling reversed the effect of palmitylcarnitine, changing its effect from causing an oxidation of extramitochondrial NADH to a more rapid reduction of extramitochondrial NAD.

The results indicated that the energy state of the mitochondria is more important for the direction of transfer of reducing equivalents through the mitochondrial membrane, than is the mitochondrial NADH/NAD ratio. This was confirmed by the effect of ATP. The effect of rotenone could be reversed by addition of ATP alone.

An active aspartate-malate shuttle is needed for the oxidation of extramitochondrial NADH [6], but in an equilibrium system like the one used here, a selective oxidation of α-ketoglutarate would be sufficient to give increased extramitochondrial NADH and an apparent reversal of the shuttle. However, experiment II of Table I shows that 1 mM arsenite (which completely blocks the oxidation of α-ketoglutarate)

only partially inhibited the formation of extramitochondrial NADH in the presence of rotenone, and it did not prevent a rapid NADH formation in the presence of palmitylcarnitine, rotenone and dinitrophenol. Other experiments showed that both α-ketoglutarate and aspartate decreased in concentration when rotenone or rotenone plus dinitrophenol were added. Thus a true reversal of the shuttle took place (see also Figs 2 and 4).

These results correspond nicely with studies by Robinson [5] who studied variations in the redox state of intramitochondrial NAD(P) under similar conditions. In the presence of malate, aspartate, α-ketoglutarate, glutamate and rotenone, he found that succinate, ATP or oxygen caused a reduction of mitochondrial NAD(P). Under similar conditions Table I shows oxidation of extramitochondrial NADH. The reduction of mitochondrial NAD(P) described by Robinson was reversed by uncouplers or anaerobiosis.

The experiments of Table I show an energy-dependent uptake of reducing equivalents into the mitochondria. The further disposal of the reducing equivalents inside the mitochondria is probably also energy-requiring, since we have previously found that palmitylcarnitine oxidation in the presence of rotenone is more rapid in coupled than in uncoupled mitochondria [12]. The exact mechanism of this removal is unknown. To eliminate this energy-requiring removal of NADH from the mitochondria themselves as a likely rate-limiting step, all further experiments were performed with acetoacetate and/or β-hydroxybutyrate in the medium. Rotenone and arsenite were also added to exclude interference from α-ketoglutarate de-

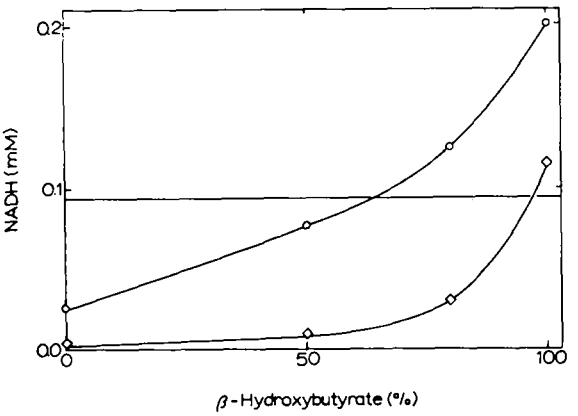

Fig. 1. The effect of a variable β-hydroxybutyrate/acetoacetate ratio on the extramitochondrial NADH/NAD ratio, in the presence of ATP or dinitrophenol. Malate, 2.5 mM; glutamate, 2.5 mM; NAD, 1.25 mM; malate dehydrogenase, 250 units; and glutamate oxaloacetate transaminase, 50 units; were preincubated for about 30 min in imidazol/HCl buffer (pH 7.4), 35 mM; and KCl, 75 mM. After addition of rotenone, 12 μM; sodium arsenite, 1 mM; and a total of 5 mM L-β-hydroxybutyrate plus acetoacetate (the hydroxybutyrate varying from 0 to 100 %); mitochondria (7.5 mg of protein) were added and the incubation was continued for 6 min. Total volume was 4 ml and the temperature was 36 °C. During the preincubation period, an equilibrium concentration of 94 mM NADH had been formed (indicated by the straight line). Nearly identical concentrations of aspartate and ketoglutarate had also been formed. ○--○, NADH concentration after 6 min in vessels incubated with dinitrophenol, 75 μM; ◇—◇, NADH concentration after 6 min in vessels incubated with ATP, 2.5 mM.

hydrogenase and the electron transport chain. The ketone bodies are assumed to be freely permeable through the mitochondrial membrane. They will therefore impose a certain NADH/NAD ratio on the rotenone-arsenite-inhibited mitochondria and thus permit the study of energy effects on the malate-aspartate shuttle.

Fig. 1 shows how a variable β-hydroxybutyrate/acetoacetate ratio affects the extramitochondrial NADH/NAD ratio in an uncoupled and in a high energy state of the mitochondria. In the presence of ATP, the extramitochondrial NAD became more oxidized than when the mitochondria were uncoupled with dinitrophenol at all β-hydroxybutyrate/acetoacetate ratios. It was calculated that the initial NADH/NAD ratio in Fig. 1 corresponded to approximately 70 % reduction of the ketone bodies. When the added ketone bodies were 50 % reduced, the extramitochondrial NADH/NAD ratio decreased, whereas it was increased if the added ketone bodies were 80 % reduced. Thus in uncoupled mitochondria, the ketone bodies seem to equilibrate freely with the extramitochondrial NADH/NAD via the malate-aspartate shuttle, while this was not the case in energized mitochondria, where pure β-hydroxybutyrate was required to give a slow reduction of the extramitochondrial NAD. That the equilibrium occurred mainly via the shuttle is shown by the effect of aminooxyacetate in Figure 2. This inhibitor of glutamate oxaloacetate transaminase almost completely prevented the reduction of extramitochondrial NAD by β-hydroxybutyrate in uncoupled mitochondria.

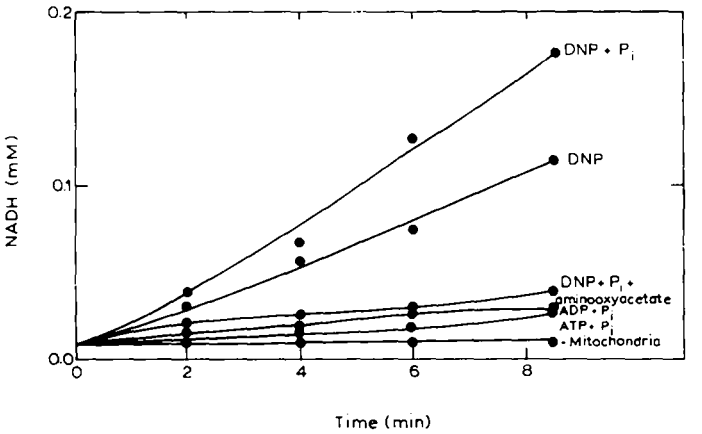

Fig. 2. The effects of dinitrophenol (DNP), 100 μM; phosphate (P_i), 2.5 mM; ATP, 2.5 mM; ADP, 2.5 mM; and of aminooxyacetate, 2.5 mM; on the reduction of extramitochondrial NAD by β-hydroxybutyrate. Malate, 1.67 mM glutamate, 1.67 mM; aspartate, 0.83 mM; α-ketoglutarate, 0.83 mM; DL-β-hydroxybutyrate, 3.33 mM; malate dehydrogenase, 500 units; glutamate oxaloacetate transaminase, 80 units, were preincubated for approximately 30 min in imidazole/HCl buffer (pH 7.4, 25 mM); and KCl, 80 mM. After addition of rotenone, 8 μM; arsenite, 1 mM; and other additions as shown, rat liver mitochondria (19 mg of protein) were added and samples taken after the time intervals shown. Total volume was 6 ml and the temperature was 36 °C.

Fig. 2 shows that egress of reducing equivalents via the malate aspartate shuttle must be slow in energized mitochondria. In this experiment the initial extramitochondrial equilibrium concentration of NADH was kept much lower than in previous experiments by addition of aspartate and α-ketoglutarate to the system

(initial NADH/NAD ratio approximately 1/200). Again uncoupling was required to give rapid NADH formation. This formation was stimulated by phosphate, as shown also in Table I. This table also shows that phosphate stimulates the uptake of reducing equivalents in an energized state. These effects of phosphate are in accordance with observations by Williamson et al. [13], who found that phosphate stimulates aspartate transport through the mitochondrial membrane.

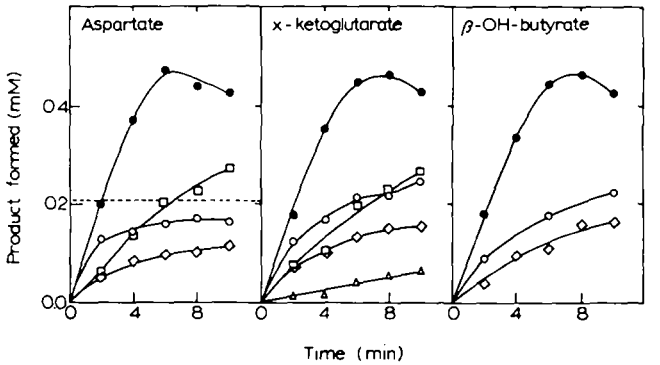

Fig. 3. The effect of energy on the reaction glu + mal + acetoacetate → asp + α-ketoglut + β-hydroxybutyrate in isolated rat liver mitochondria. Rat liver mitochondria (12 mg of protein) were incubated with imidazole/HCl buffer (pH 7.4), 22 mM; KCl, 75 mM; phosphate, 2 mM; rotenone, 10 μM; arsenite 1 mM; malate, 3 mM; glutamate, 3 mM; acetoacetate, 3 mM. Total volume was 10 ml and incubation temperature was 36 °C. Other additions: ● — ●, ATP, 3 mM; ○ — ○, ATP + oligomycin; ◇ — ◇, DNP, 0.1 mM; □ — □, ATP + DL-hydroxybutyrate, 12 mM; - - -, Equilibrium concentration of aspartate in an incubation where malate dehydrogenase (90 units), glutamate oxaloacetate transaminase (20 units), β-hydroxybutyrate dehydrogenase (2.5 units); and NAD, 0.2 mM, substituted the mitochondria. △ — △, ATP, 3 mM; no malate added.

Fig. 3 shows how ATP or uncoupling affect the conversion of malate, glutamate and acetoacetate to aspartate, α-ketoglutarate and β-hydroxybutyrate, i.e., the mitochondrial part of malate-aspartate shuttle for uptake of reducing equivalents. Aspartate, α-ketoglutarate, and β-hydroxybutyrate were formed in approximately equimolar amounts. When malate was omitted from the incubation mixture only a small amount of α-ketoglutarate was formed, showing that most of the α-ketoglutarate was formed by transamination, not by glutamic acid dehydrogenase. Evidently, all the reaction products were formed to a greater extent in energized mitochondria (ATP added). In the energized system, the concentration of all three products were considerably higher than the equilibrium concentrations of aspartate when a soluble system was permitted to run to equilibrium after addition of malic dehydrogenase, glutamate oxaloacetate transaminase, soluble β-hydroxybutyrate dehydrogenase and catalytic amounts of NAD. The reaction was inhibited by oligomycin, showing that energization of the mitochondrial membrane was involved in the reaction. Another experiment (not shown) showed that the medium concentration of aspartate obtained, depended on the initial ATP/ADP ratio added to the incubation mixture.

Table I and Figs 1 and 2 indicated that the malate-aspartate shuttle is reversible, but only slowly, if at all, in energized mitochondria. However, when experiments

were undertaken to study the effect of energy state on the mitochondrial part of the reversal (i.e., the conversion of aspartate, α-ketoglutarate and β-hydroxybutyrate to glutamate and acetoacetate), we found no significant difference in malate formation in uncoupled and energized mitochondria (not shown). Further tests showed that incubation of rat liver mitochondria with aspartate and α-ketoglutarate led to a significant formation of oxaloacetate. The malate formed, therefore, might represent extramitochondrially-formed oxaloacetate (by transaminase from damaged mitochondria), subsequently taken up and reduced by the mitochondria, i.e. by a mechanism not representing a reversal of the malate-aspartate shuttle. Evidently the complete shuttle system (Table I, Figs 1 and 2) reacted differently to de-energization than a system omitting NAD and the shuttle enzymes. The difference proved to be due to the absence of glutamate from the latter.

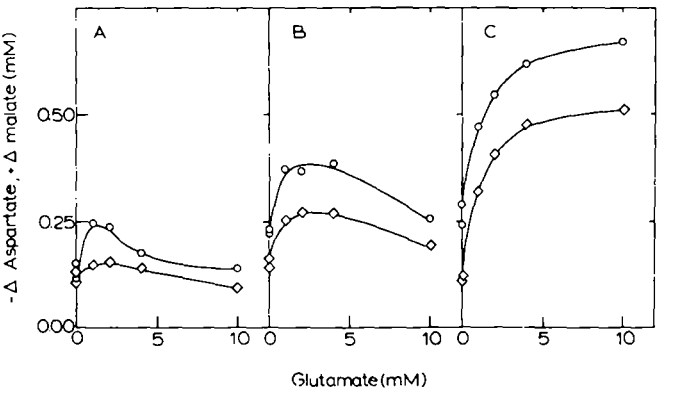

Fig. 4. The effect of glutamate on the reaction aspartate + α-ketoglutarate + β-hydroxybutyrate → malate + glutamate + acetoacetate in isolated rat liver mitochondria. Rat liver mitochondria (4 mg of protein) were incubated with imidazole/HCl buffer (pH 7.4, 30 mM); KCl, 75 mM; phosphate, 2 mM; rotenone, 10 μM; arsenite, 1 mM; aspartate, 1 mM; α-ketoglutarate, 1 mM; and DL-β-hydroxybutyrate, 3 mM. Total volume was 2.5 ml, incubation time was 5 min and the temperature was 36 °C. Other additions: (A) ATP, 2 mM (B) ADP, 2 mM (C) DNP, 80 μM. Glutamate, 0–10 mM as shown. ○ – ○, aspartate disappearance; ◇ – ◇, malate formation.

Fig. 4 shows that in the absence of glutamate, malate formation was about equal in energized and uncoupled mitochondria (although there was some difference in aspartate disappearance). Part of this malate formation most likely was the result of extramitochondrial oxaloacetate formation, followed by a mitochondrial uptake and reduction. However, with the addition of glutamate, the disappearance of aspartate and the formation of malate were stimulated and this was increased in the uncoupled mitochondria. The stimulation by uncoupling in this reaction has also been observed by De Haan and Oestreicher [14]. The stimulation by glutamate is in accordance with Azzi, Chapell and Robinson [15], who observed that glutamate stimulates aspartate entry into mitochondria. The control without malate added, in Fig. 3, shows that the conversion of glutamate to α-ketoglutarate is extremely slow

under the conditions used. An increased formation of oxaloacetate by extramitochondrial transaminase, therefore cannot explain the results. Thus, Fig. 4 indicates that aspartate can be taken up by rat liver mitochondria, even in a high energy state, but the rate is greatly stimulated by de-energization. No corresponding effect of α-ketoglutarate on the rate of malate formation was observed.

DISCUSSION

The present studies confirm that there is an active transport of extramitochondrial, reducing equivalents into the mitochondria via the malate-aspartate shuttle. As anticipated, an energy input in the system is required in arsenite-rotenone inhibited mitochondria. This energy could be furnished either by ATP (inhibited by oligomycin) or by flavoprotein-linked substrates, such as succinate and palmityl-CoA (inhibited by uncouplers). Our experiments do not show the exact mechanism of this active transport. Acetoacetate and β-hydroxybutyrate are probably in direct equilibrium with the mitochondrial NADH and NAD. It is unlikely that our results can be explained by active transport of these carboxylic acids. In accordance with this, Robinson [5] found no effect of ATP on the NADH/NAD ratio in the presence of acetoacetate and β-hydroxybutyrate.

Malate is actively taken up by mitochondria in exchange for phosphate which is, in turn, taken up in exchange for hydroxyl ions. The uptake of malate therefore appears to depend on a pH difference across the mitochondrial membrane. This active uptake might represent an energy input in the aspartate-malate shuttle. However, the other dicarboxylic acid involved, α-ketoglutarate, is taken up or transported out, in exchange for malate. It seems likely, therefore, that a difference in pH across the mitochondrial membrane will have a similar effect on the distribution of α-ketoglutarate and malate, and any net effect on the aspartate-malate shuttle will be nullified. These considerations leave us with the transport of aspartate and glutamate to explain the operation of the aspartate-malate shuttle. Glutamate is taken up by mitochondria in exchange for hydroxyl ions or in exchange for aspartate [16]. Thus, a difference in pH across the mitochondrial membrane (alkaline inside) may increase glutamate uptake, which will represent an energy input. However, perhaps more important is a recent observation by LaNoue et al. [9], who have found that glutamate-aspartate exchange can take place only with a simultaneous net transfer of charges across the mitochondrial membrane. The exchange transport of these amino acids, therefore is electrogenic and the chemical equilibrium of the overall reaction (e.g. as in the experiment of Fig. 3) is displaced by the potential across the mitochondrial membrane. Consequently a skewed redox situation, across the inner mitochondrial membrane, which has the more reduced inside is created. Recent experiments by Williamson et al. [2] and Berry et al. [4] have shown that rotenone or amytal inhibits the oxidation of extramitochondrial NADH by 50–60 % in liver cells. They conclude, that up to 50 % of the reducing equivalents may be taken up by the mitochondria via the α-glycerophosphate-dihydroxyacetonephosphate shuttle. Our results show that this may represent an overestimation of the capacity of the glycerophosphate shuttle. Isolated energized mitochondria can oxidize some NADH via the aspartate-malate shuttle or from β-oxidation [12], even in the presence of rotenone. How this NADH is oxidized is unknown, but it shows that the use of rotenone (or amytal) for

the estimation of the capacity of the α-glycerophosphate shuttle for reducing equivalents is not reliable.

Berry [17] found that rotenone inhibits reduction of pyruvate to lactate in liver cells and suggested that the transfer of reducing equivalents from the mitochondria to the extramitochondrial cytoplasm is an energy-requiring process. Meijer and Williamson [18] have suggested that this transfer may occur via malate which is converted to phosphoenolpyruvate and pyruvate outside the mitochondria. Subsequently the pyruvate is converted back to malate via pyruvate carboxylase and malate dehydrogenase, inside the mitochondria.

Our results show that a rapid efflux of reducing equivalents via the malate-aspartate shuttle requires both uncoupling and inhibition of NADH oxidation in the mitochondria. However, the detectable glutamate-stimulated aspartate entry into energized mitochondria (Fig. 4) suggests that the shuttle is not completely irreversible in the intact cell. The residual reduction of pyruvate to lactate in the presence of rotenone, observed by Berry [17] and by Meijer and Williamson [18] may therefore, at least in part, be due to a reversed malate-aspartate shuttle. Apart from this possibility, the mechanism of inhibition of pyruvate reduction by rotenone remains to be established. The slowness of the reversed shuttle in energized mitochondria makes it likely that an additional mechanism, for the transport of reducing equivalents out of mitochondria, is needed.

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