Properties of the branched-chain 2-hydroxy acid/2-oxo acid shuttle in mouse spermatozoa*

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Operation of the branched-chain 2-hydroxy acid/2-oxo acid shuttle for the transfer of reducing equivalents in mitochondria of mouse spermatozoa was studied *in vitro* in reconstituted systems. Results show that the branched-chain 2-oxo acids within the mitochondria are offered several metabolic pathways. (a) Decarboxylation: mouse sperm mitochondria possess high branched-chain 2-oxo acid decarboxylase activity. (b) Recycling to the cytosol by using a transport system which can be inhibited by α -cyano-3-hydroxycinnamate and pH 6.8. (c) Transamination to the corresponding amino acids: experiments presented indicate that leucine formed from 4-methyl-2-oxopentanoate may pass to the external phase, re-initiating the cycle. These two last possibilities would allow autocatalytic operation of the shuttle. The branched-chain 2-hydroxy acids apparently do not utilize the monocarboxylate carrier to penetrate the mitochondria.

INTRODUCTION

Studies in this laboratory have afforded evidence on the operation, in mouse spermatozoa, of a shuttle system involving the redox couple branched-chain 2-hydroxy acid/2-oxo acid and LDH X, the isoenzyme X or C₄ of lactate dehydrogenase (Blanco *et al.*, 1976; Gerez de Burgos *et al.*, 1978; Burgos *et al.*, 1982).

The 2-oxo acids formed in the cytosol by transamin-

ation of branched-chain amino acids are reduced by LDH X, thereby oxidizing cytoplasmic NADH. The 2-hydroxy acids thus formed penetrate into the mitochondria, where they are oxidized back to 2-oxo acids by LDH X. In this reaction, hydrogen atoms are transferred to intramitochondrial NAD⁺.

Observations *in vitro* showed that the system can be reconstituted with branched-chain 2-hydroxy acids, 2-oxo acids or the corresponding amino acids as initial substrates. Conditions in which it is possible to demon-

Scheme 1. The branched-chain 2-hydroxy acid/2-oxo acid shuttle for the transfer of reducing equivalents in mouse sperm mitochondria

Abbreviations used: LDH X, isoenzyme X of lactate dehydrogenase (EC 1.1.1.27); LAT, leucine aminotransferase (EC 2.6.1.6); NEM, *N*-ethylmaleimide.

* Dedicated to Dr. Luis F. Leloir on the occasion of his 80th birthday, 6 September 1986.

strate cyclic operation of the shuttle have been reported by Burgos *et al.* (1982).

The present paper reports further studies on reconstituted systems showing the possible alternative pathways for the 2-oxo acids within the mitochondria and the involvement of the monocarboxylate carrier in the function of the shuttle.

EXPERIMENTAL

Materials

Mitochondria. 'Sperm-type' mitochondria were obtained from testis of adult albino Swiss mice as described by Machado de Domenech *et al.* (1972). Mouse liver mitochondria were isolated in 0.25 M-sucrose/20 mM-Tris/HCl, pH 7.4, by the method of Hogeboom (1955). Heart mitochondria were prepared by the technique described by Holian *et al.* (1977).

Enzymes. LDH X was purified from adult mouse testes by the method of Goldberg (1972). Branched-chain aminoacid aminotransferase (leucine aminotransferase, EC 2.6.1.6; LAT) was purified from mouse kidney as indicated by Aki *et al.* (1968). Units of enzyme activity are as defined by Burgos *et al.* (1982).

Chemicals. All chemicals used were obtained from Sigma (St. Louis, MO, U.S.A.), except α -cyano-3-hydroxycinnamic acid, purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Methods

Reconstituted systems *in vitro*. Two different systems were utilized: (a) with 4-methyl-2-oxopentanoate (α -ketoisocaproate) and NADH (at concentrations given in the Results and discussion section), 5 units of LDH X, 25 mM-imidazole/HCl buffer, pH 7.4, and 75 mM-KCl in a final volume of 3 ml (Burgos *et al.*, 1982); (b) an adaptation of the conditions described by Bremer & Davis (1975) for the malate/aspartate shuttle. The preincubation mixture was composed of DL-2-hydroxy-4-methylpentanoate (DL- α -hydroxyisocaproate) (concentrations are given in Results and discussion section), 1.7 mM-NAD⁺, 5 units of LDH X, 3.3 mM-L-glutamate, 5 units of LAT, 25 mM-imidazole/HCl buffer, pH 7.4, and 75 mM-KCl in a final volume of 3 ml.

The mixtures were left at room temperature (20–23 °C) for 20 min and then the mitochondria were added. Tubes were incubated for 10 min in a water bath at 37 °C. Blanks comprised the same mixture, except that 0.32 M-sucrose was substituted for the mitochondrial suspension. Formation and/or disappearance of NADH was measured after enzyme inactivation in a boiling-water bath during 90 s. Tubes were immediately cooled on ice and centrifuged for 10 min at 15000 g. Supernatants were used for determinations of NADH and 2-oxo acids.

NADH and 4-methyl-2-oxopentanoate assays and control of mitochondrial integrity were performed as previously described (Burgos *et al.*, 1982).

In all experiments, controls were run with NADH as the only substrate, preincubated under the same conditions as described for the experimental tubes. These controls showed that incubation for 10 min at 37 °C with mitochondria produced no oxidation of external NADH.

Mitochondria used in all experiments gave a percentage of integrity higher than 95% (see Burgos *et al.*, 1982). As previously reported (Burgos *et al.*, 1982), addition of 0.01 μ M-rotenone or 0.016 mM-antimycin completely inhibits the oxidation of extramitochondrial NADH in the system. These observations demonstrate that practically all the NADH utilization on addition of mitochondria is confined to the shuttle.

Determination of 2-oxoglutarate and L-glutamate. The concentration of 2-oxoglutarate was measured as indicated by Bergmeyer & Bernt (1974).

L-Glutamate was determined in the incubation mixture as follows: to 2 ml of the preparation was added 0.5 ml of 2 M-HClO₄. After centrifugation for 10 min at 15000 g, the supernatant was neutralized with 5 M-K₂CO₃ and centrifuged again for 10 min at 15000 g. L-Glutamate was assayed in the supernatant by the method described by Bernt & Bergmeyer (1974).

2-Oxo acid decarboxylase assay. Determinations of pyruvate dehydrogenase (lipoamide) (EC 1.2.4.1) and 2-oxoisovalerate dehydrogenase (lipoamide) (EC 1.2.4.4) were carried out at 37 °C by the method of Gubler (1961) as modified by Sullivan *et al.* (1976). Enzymic activity was measured spectrophotometrically by following the reduction of ferricyanide at 420 nm in 10 mm-light-path cuvettes. The reaction mixture contained 20 mM-sodium phosphate buffer, pH 7.4, 2.7 mM-K₃Fe(CN)₆, 5 mM-MgCl₂, 0.54 mM-Na₂EDTA, 3 mg of mitochondrial protein, the 2-oxo acid at saturating concentration (5 mM), and sucrose solution (0.25 M for liver and heart, 0.32 M for sperm mitochondria) up to a final volume of 3.7 ml. The reaction was started by addition of substrate. Controls for endogenous reaction were performed in identical conditions (except addition of substrate) and the value obtained was subtracted from the experimental results.

Determinations were also carried out after incubating the enzyme preparation for 30 min in the buffer system described by Odessey (1980) with and without 1.6 mM-ATP added to the mixture. In these cases, enzymic activity was determined as described above. The activity is expressed in nmol of Fe(CN)₆³⁻ reduced/min. In our conditions, an absorption change (Δ_{420}) of 0.001 corresponds to 1 nmol of Fe(CN)₆³⁻ reduced.

Protein determination. Total protein was measured in the preparations by the technique of Gornall *et al.* (1949).

RESULTS AND DISCUSSION

Effect of inhibition of the monocarboxylate carrier on the shuttle activity

It was previously reported (Burgos *et al.*, 1982) that the shuttle could be reconstituted with 2-oxo acid, NADH and LDH X. By using 4-methyl-2-oxopentanoate as initial substrate, at concentrations lower than those of NADH, the 2-oxo acid was completely reduced to 2-hydroxy acid during preincubation. However, the system was able to oxidize extra NADH on addition of mitochondria. This observation demonstrated the cyclic operation of the shuttle, since the results could only be explained if intramitochondrially formed 2-oxo acids returned to the cytosol (Burgos *et al.*, 1982).

In order to study whether the movement of 2-oxo acids and 2-hydroxy acids across the mitochondrial membranes depends on specific transport mechanisms, experiments were carried out on the effect, on the shuttle activity, of α -cyano-3-hydroxycinnamate, a potent inhibitor of pyruvate transport (Halestrap, 1978).

Expt. A (Table 1) was performed with a low concentration of 4-methyl-2-oxopentanoate. After equilibrium, the 2-oxo acid and NADH are consumed in the mixture in stoichiometric amounts. As no 2-oxo acid is left in the medium, extra oxidation of NADH produced after addition of mitochondria must involve recycling of 2-oxo acid from the organelles. In the presence of 0.5 mM- α -cyano-3-hydroxycinnamate (Expt. B), oxidation of external NADH on addition of mitochondria is completely blocked. When the concentration of the substrate was increased to allow for an excess after preincubation (Expt. D), addition of the inhibitor did not modify the oxidation of external NADH (Expt. E, Table 1).

These results indicate that the effect of α -cyano-3-hydroxycinnamate is restricted to 4-methyl-2-oxopentanoate transport, blocking the efflux of the 2-oxo acid from the mitochondria, whereas the influx of 2-hydroxy acid into the organelles is apparently not modified.

A similar interpretation can be offered for results of experiments performed at two different pH values (7.4 and 6.8). It is known that pyruvate efflux from rat liver mitochondria is highly dependent on pH. At pH 6.8, the efflux is practically blocked (Halestrap, 1978). Results presented in Table 1 show similar pH-sensitivity for the transport of 2-oxo acid in mouse sperm mitochondria. When a relatively low concentration of 4-methyl-2-oxopentanoate is used (Expt. C), the system is inactive at pH 6.8. This indicates that efflux of the 2-oxo acid from mitochondria is suppressed at pH 6.8. In Expt. F, with a higher amount of substrate, the activity of the system is much less affected by the lower pH. In these conditions, there is an excess of the 2-oxo acid in the external phase,

and efflux from the mitochondria is not needed for oxidation of extra NADH, provided that the 2-hydroxy acid penetrates into the organelle and displaces equilibrium towards further reduction of 2-oxo acid.

Activity of 2-oxo acid decarboxylase in mouse mitochondria

One of the possible metabolic fates of the branched-chain 2-oxo acids formed within the mitochondria during operation of the shuttle is degradation *in situ*, which involves a first step of oxidative decarboxylation.

To compare the ability of mitochondria from different tissues to decarboxylate branched-chain 2-oxo acids, determinations of decarboxylase activity were performed in heart, liver and sperm-type mitochondria. Table 2 presents the results expressed as specific activity. Relative activities of liver mitochondria with pyruvate, 3-methyl-2-oxobutanoate (α -ketoisovalerate), 4-methyl-2-oxopentanoate and 3-methyl-2-oxopentanoate (α -keto- β -methylvalerate) are very similar to those reported by Sullivan *et al.* (1976). Sperm-type mitochondria showed relatively higher activity with the branched-chain 2-oxo acids than did heart and liver mitochondria.

Odessey (1980) and Hughes & Halestrap (1981) have demonstrated that the branched-chain 2-oxo acid dehydrogenase, as well as pyruvate dehydrogenase, is inhibited by phosphorylation. Thus conditions of assay must be selected to ensure activation of the enzymes.

We repeated the above-mentioned determinations by adding 1.6 mM-ATP, and found 60% inhibition of the enzyme in liver mitochondria and 70% of that in sperm-type mitochondria. We have also performed the assays after 30 min incubation in the buffer systems proposed by Odessey (1980). Liver preparations showed only about 30% activation, whereas those of sperm-type mitochondria did not change. These results suggest that the enzymes are activated in the assay conditions of Sullivan *et al.* (1976).

Table 1. Effect of inhibition of the monocarboxylate carrier on the activity of the shuttle in mouse spermatozoa

After preincubation, sperm-type mitochondria were added in an amount corresponding to 2.64 mg of total protein. Values are means of duplicate determinations. Results are from one experiment representative of five performed in similar conditions. Δ NADH is the difference between the amount of NADH present in the mixture after preincubation, and the final amount present after 10 min incubation with mitochondria.

Experiment	Substrate	Amount initially added (nmol)	Amount after preincubation (nmol)	Δ NADH (nmol)
A pH 7.4	4-Methyl-2-oxopentanoate	300	0	
	NADH	1656	1354	-197
B pH 7.4: same as A + 0.5 mM- α -cyano-3-hydroxycinnamate	4-Methyl-2-oxopentanoate	300	0	
	NADH	1656	1358	+1
C: same as A, but pH 6.8	4-Methyl-2-oxopentanoate	300	0	
	NADH	1456	1156	-1
D pH 7.4	4-Methyl-2-oxopentanoate	1500	230	
	NADH	1413	143	-124
E pH 7.4: same as D + 0.5 mM- α -cyano-3-hydroxycinnamate	4-Methyl-2-oxopentanoate	1500	230	
	NADH	1412	145	-120
F: same as D, but pH 6.8	4-Methyl-2-oxopentanoate	1500	586	
	NADH	1507	593	-113

Table 2. Activity of 2-oxo acid decarboxylase in mouse heart, liver and sperm-type mitochondria

Values are expressed in nmol of $\text{Fe}(\text{CN})_6^{3-}$ reduced/mg of total protein and represent means of duplicate determinations on three different samples. Numbers in parentheses are standard deviations.

Substrate	Specific activity		
	Heart	Liver	Sperm-type
Pyruvate	45.25 (0.93)	51.5 (0.49)	65.0 (0.65)
4-Methyl-2-oxopentanoate	14.7 (1.6)	13.7 (1.4)	47.3 (3.4)
3-Methyl-2-oxopentanoate	12.9 (1.3)	20.0 (1.9)	45.2 (3.7)
3-Methyl-2-oxobutanoate	16.5 (1.5)	21.2 (2.0)	43.2 (3.5)

Table 3. Studies of leucine recycling in the shuttle system of mouse spermatozoa

After preincubation, sperm-type mitochondria were added in an amount corresponding to 2.82 mg of total protein for Expts. A and B, and 5.38 mg of total protein for Expt. C. Values are means of duplicate determinations. Results are from one experiment representative of four performed in similar conditions. In Expt. C, determinations of L-glutamate content in mitochondria gave a value of 17.3 nmol/mg of protein, which can account for the amino groups needed for glutamate formed intramitochondrially. Δ is the difference between the amount present in the mixture after preincubation and the final amount present after 10 min incubation with mitochondria.

Experiment	Substrate	Amount initially added (nmol)	Amount after preincubation (nmol)	Δ (nmol)
A: mixture as indicated in text, + 0.5 mM- α -cyano-3-hydroxycinnamate	4-Methyl-2-oxopentanoate	600	0	
	2-Oxoglutarate	730	263	-116
	NADH	2290	1191	-134
	L-Glutamate	0	0	+27
B: same as A + 1 mM-arsenite	4-Methyl-2-oxopentanoate	600	0	
	2-Oxoglutarate	730	270	-70
	NADH	2303	1200	-328
	L-Glutamate	0	0	+28
C: same as A + 0.6 mM-NEM	4-Methyl-2-oxopentanoate	600	0	
	2-Oxoglutarate	500	350	-305
	NADH	2793	2005	-598
	L-Glutamate	0	0	+80

Recycling of leucine

Since efflux of 4-methyl-2-oxopentanoate from mitochondria can be blocked by α -cyano-3-hydroxycinnamate, addition of the inhibitor would force the intramitochondrial 2-oxo acid to follow alternative pathways, e.g. oxidative decarboxylation or transamination to leucine (it is known that LAT has the same subcellular localization as LDH X, being present in cytosol and in the matrix of mitochondria of mouse spermatozoa; Montamat *et al.*, 1978). Leucine may pass to the 'cytosol' and be transaminated back to 2-oxo acid in the external phase.

With a preincubation mixture containing a low amount of 4-methyl-2-oxopentanoate with respect to that of NADH, α -cyano-3-hydroxycinnamate to block the monocarboxylate carrier, and LAT and 2-oxoglutarate to allow transamination, it was possible to demonstrate efflux of leucine from mitochondria (Table 3).

The system was preincubated at room temperature with the following ingredients: 0.2 mM-4-methyl-2-oxopentanoate, 0.77 mM-NADH, 5 units of LDH X, 5 units of LAT, 0.5 mM- α -cyano-3-hydroxycinnamate and the imidazole/HCl buffer. After 20 min, 0.25 mM-2-oxogluta-

rate and mitochondria were added simultaneously and incubated at 37 °C for 10 min, as indicated in the Experimental section. As LDH X catalyses the reduction of 4-methyl-2-oxopentanoate and of 2-oxoglutarate (Blanco *et al.*, 1976), this second 2-oxo acid is added after equilibrium and complete consumption of the former are reached. On the other hand, the assay with LDH X measures the amount of both 2-oxo acids present in the mixture. The concentration of 4-methyl-2-oxopentanoate, then, is calculated by difference between total 2-oxo acids determined with LDH X and the amount of 2-oxoglutarate assayed with glutamate dehydrogenase.

Results are presented in Table 3. The originally added 4-methyl-2-oxopentanoate is completely used up during preincubation. After addition of mitochondria, extra NADH is oxidized (Expt. A). Part of this oxidized NADH corresponds to that participating in the LDH X-catalysed reduction of 2-oxoglutarate. Since there are many possible fates for 2-oxoglutarate (reduction by LDH X, transamination with leucine, influx or efflux to or from the mitochondria), it is impossible to ascertain the amount of NADH oxidized which can be accounted

for by reduction of 2-oxoglutarate. If we add 1 mM-arsenite to the preincubation mixture (Table 3, Expt. B), the amount of extramitochondrial NADH oxidized by the system is higher. As arsenite inhibits the activity of mitochondrial 2-oxo acid decarboxylase, more 2-oxo acid is available for transamination within the mitochondria and more leucine can return to the cytosol. The result of this experiment suggests that 4-methyl-2-oxopentanoate is being generated in the external medium, allowing oxidation of extra NADH. Since the monocarboxylate carrier is inhibited by α -cyano-3-hydroxycinnamate, the only possible source of the 2-oxo acid is transamination of leucine.

Leucine recycling is confirmed by Expt. C (Table 3). The system is reconstituted as for Expt. A, plus addition of 0.6 mM-NEM to inhibit L-glutamate transport (Bradford & McGivan, 1973). After addition of mitochondria, L-glutamate is generated in the external phase. This can only be explained if leucine egresses from the mitochondria and transaminates with 2-oxoglutarate in the cytosol. The amount of glutamate found in the external phase is lower in Expts. A and B than in Expt. C. It is possible that part of the glutamate formed or arriving in the external medium can penetrate into the mitochondria when no inhibitor of transport is present.

Effect of glutamate on the activity of the shuttle

It had been demonstrated that the system reconstituted with L-glutamate, LAT, 2-hydroxy acid, NAD⁺ and LDH X was able to oxidize preformed NADH on addition of sperm mitochondria (Burgos *et al.*, 1982). By using DL-2-hydroxy-4-methylpentanoate as initial substrate at 6.25, 12.5, 25.0, 37.5 and 50.0 mM concentrations, it was observed that NADH formed during preincubation of the cytosolic moiety of the system increased with substrate concentration. NADH oxidized after addition of mitochondria was highest when the starting substrate concentrations were 25.0 or 37.5 mM. When the same experiments were performed without L-glutamate and LAT in the preincubation mixture, the activity was higher than that of the complete system, at all substrate concentrations used. Although preformed NADH did not change whether L-glutamate and LAT were present or not, there was a marked difference in the NADH oxidized after addition of mitochondria.

The experiments shown in Table 4 were performed with 25.0 mM-DL-2-hydroxy-4-methylpentanoate as initial substrate. Omission of glutamate and LAT from the

mixture markedly increases the specific activity of the system.

The inhibitory action of glutamate could be explained by assuming that, in the complete system, this amino acid can be transported into the mitochondria, where it is oxidized by glutamate dehydrogenase, thereby competing with the shuttle system for NAD⁺ within the organelle. To investigate this possibility, we studied the effect, on the shuttle activity, of NEM, an inhibitor of glutamate transport in mitochondria. The cytosolic portion of the system was prepared with the complete mixture containing L-glutamate and LAT. NEM was added at 0.15 and 0.60 mM concentrations. Assays performed after incubating the enzymes for 20 min with 0.60 mM-NEM demonstrated no effect on LDH X and LAT. As shown in Table 4, the amount of preformed NADH was not modified by NEM.

The amount of NADH oxidized after addition of mitochondria was higher when NEM was present. Activity with 0.60 mM-NEM was practically the same as that of the system without L-glutamate and LAT. These results support the above-mentioned assumption about the mechanism of inhibition by glutamate.

The lactate/pyruvate couple

After our proposal of the branched-chain 2-hydroxy acid/2-oxo acid shuttle in mouse spermatozoa (Blanco *et al.*, 1976), the existence of a lactate/pyruvate shuttle in rabbit spermatozoa was proposed by Storey & Kayne (1977). The intramitochondrial location of LDH X, initially demonstrated in our laboratory (Machado de Domenech *et al.*, 1970, 1972; Montamat & Blanco, 1976), was confirmed by other investigators (Storey & Kayne, 1977; Van Dop *et al.*, 1977). Studies in Lardy's laboratory showed utilization of pyruvate and lactate by intramitochondrial LDH X in bovine spermatozoa (Van Dop *et al.*, 1977, 1978; Hutson *et al.*, 1977).

Our observations indicate that the redox couple lactate/pyruvate is not utilized by the LDH X shuttle in mouse spermatozoa. Previous results with reconstituted systems *in vitro*, using lactate and NAD⁺ as initial substrates, showed no oxidation of preformed NADH on addition of mitochondria. On the contrary, there was an increase in the reduced coenzyme (Burgos *et al.*, 1982). As the reaction $\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$ is highly displaced to the right, the system *in vitro* containing pyruvate and NADH as original substrates

Table 4. Effect of L-glutamate on activity of the shuttle system in mouse spermatozoa

After preincubation, sperm-type mitochondria were added in an amount corresponding to 4.88 mg of total protein. The preincubation mixture was composed of 25.0 mM-DL-2-hydroxy-4-methylpentanoate, 1.7 mM-NAD⁺, 5 units of LDH X, 3.3 mM-L-glutamate, 5 units of LAT, 25 mM-imidazole/HCl buffer, pH 7.4, and 75.0 mM-KCl in a final volume of 3 ml. Values are means of duplicate determinations. Results are from one experiment representative of five performed in similar conditions. Δ NADH is as in Table 1.

Conditions	Preformed NADH (nmol)	Final NADH (nmol)	Δ NADH (nmol)	NADH oxidized (nmol/min per mg of protein)
Complete system	181.3	129.0	-52.3	1.07
System without glutamate and LAT	177.1	95.2	-81.9	1.66
Complete system + 0.15 mM-NEM	179.0	117.8	-61.2	1.25
Complete system + 0.60 mM-NEM	179.0	105.6	-73.4	1.50

produces complete oxidation of the coenzyme during preincubation when the molar concentration of pyruvate is equal to or higher than that of NADH. When pyruvate concentration is lower than that of NADH, all the substrate is used up during preincubation.

We have found that the ratio of consumption pyruvate/lactate for mouse sperm mitochondria is 21.6, whereas it is 1.6 for rabbit sperm mitochondria. Thus, when mouse sperm mitochondria are added to the preincubation mixture with the couple lactate/pyruvate, the predominant consumption of pyruvate shifts the equilibrium towards oxidation of preformed lactate and increases the NADH concentration in the external phase. On the contrary, when rabbit sperm mitochondria are added, there is oxidation of preformed NADH (N. M. Gerez de Burgos, C. Burgos, F. G. Gallina, C. E. Coronel & A. Blanco, unpublished work).

CONCLUSIONS

Data presented here afford additional evidence on the operation, in mouse sperm mitochondria, of the branched-chain 2-hydroxy acid/2-oxo acid shuttle as outlined in Scheme 1.

Results indicate the existence of the following alternative pathways for the 2-oxo acids within the mitochondria.

(a) Return to the external phase. It appears that 4-methyl-2-oxopentanoate is transported by the same carrier as pyruvate, or at least by one with similar properties (Halestrap, 1975, 1978; Mowbray, 1975). The 2-oxo acid efflux from mitochondria allows autocatalytic operation of a 'short cycle' (Scheme 1).

(b) Degradation in the mitochondria via decarboxylation. The important activity of branched-chain 2-oxo acid decarboxylase in mouse sperm mitochondria would indicate that this alternative pathway is by no means negligible. It would withdraw intermediates from the cycle.

(c) Transamination to the corresponding amino acids, which may pass to the external phase, completing the 'long cycle' (Scheme 1). Experiments *in vitro* have also shown this possibility. Amino acid recycling would feed the autocatalytic operation of the shuttle. This recycling leads to extramitochondrial production of L-glutamate. As shown in reconstituted systems, the presence of this amino acid can decrease the activity of the shuttle, suggesting that the 'long cycle' could be self-regulated.

It appears that the 2-hydroxy acids formed in the cytosol by the LDH X-catalysed reduction of branched-chain 2-oxo acids do not utilize the monocarboxylate carrier to penetrate the mitochondria. Halestrap (1975) has shown that 4-methyl-2-oxopentanoate, besides using the pyruvate carrier, is capable of crossing the liver mitochondrial membrane by free diffusion. In the conditions of our experiments, free diffusion of the branched-chain monocarboxylate in mouse sperm mitochondria appears negligible.

As reported by Coronel *et al.* (1983), the shuttle can be functional in mouse and rat spermatozoa, species whose LDH X is able to catalyse the interconversion branched-chain 2-hydroxy acid $\rightleftharpoons$ 2-oxo acid. In other species, LDH X has different substrate specificity and cannot utilize branched-chain 2-oxo or 2-hydroxy acids. However, its dual localization, in cytosol and mitochon-

drial matrix (Montamat & Blanco, 1976), enables this sperm-specific isoenzyme to participate in shuttle systems, using different redox couples in different species. In agreement with findings by Storey & Kayne (1977), observations in our laboratory indicate that the lactate/pyruvate couple is utilized by rabbit spermatozoa (N. M. Gerez de Burgos, C. Burgos, F. G. Gallina, C. E. Coronel & A. Blanco, unpublished work).

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REFERENCES

- Aki, K., Ogawa, K. & Ichihara, A. (1968) *Biochim. Biophys. Acta* **159**, 276–284
- Bergmeyer, H. U. & Bernt, E. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 3, pp. 1577–1580. Verlag Chemie, Weinheim, and Academic Press, New York
- Bernt, E. & Bergmeyer, H. U. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 1704–1708. Verlag Chemie, Weinheim, and Academic Press, New York
- Blanco, A., Burgos, C., Gerez de Burgos, N. M. & Montamat, E. E. (1976) *Biochem. J.* **153**, 165–172
- Bradford, N. M. & McGivan, J. D. (1973) *Biochem. J.* **134**, 1023–1029
- Bremer, J. & Davis, J. (1975) *Biochim. Biophys. Acta* **376**, 387–397
- Burgos, C., Coronel, C. E., Gerez de Burgos, N. M., Rovai, L. E. & Blanco, A. (1982) *Biochem. J.* **208**, 413–417
- Coronel, C. E., Burgos, C., Gerez de Burgos, N. M., Rovai, L. E. & Blanco, A. (1983) *J. Exp. Zool.* **225**, 379–385
- Gerez de Burgos, N. M., Burgos, C., Montamat, E. E., Moreno, J. & Blanco, A. (1978) *Biochem. Biophys. Res. Commun.* **81**, 644–649
- Goldberg, E. (1972) *J. Biol. Chem.* **247**, 2044–2048
- Gornall, A. G., Bardawill, C. S. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Gubler, C. J. (1961) *J. Biol. Chem.* **236**, 3112–3120
- Halestrap, A. P. (1975) *Biochem. J.* **148**, 85–96
- Halestrap, A. P. (1978) *Biochem. J.* **172**, 377–387
- Hogebloom, G. H. (1955) *Methods Enzymol.* **1**, 16–19
- Holian, A., Owen, C. S. & Wilson, D. F. (1977) *Arch. Biochem. Biophys.* **181**, 164–171
- Hughes, W. A. & Halestrap, A. P. (1981) *Biochem. J.* **196**, 459–469
- Hutson, S. M., Van Dop, C. & Lardy, H. A. (1977) *J. Biol. Chem.* **252**, 1309–1315
- Machado de Domenech, E. E., Domenech, C. E. & Blanco, A. (1970) *Arch. Biochem. Biophys.* **141**, 147–154
- Machado de Domenech, E. E., Domenech, C. E., Aoki, A. & Blanco, A. (1972) *Biol. Reprod.* **6**, 136–147
- Montamat, E. E. & Blanco, A. (1976) *Exp. Cell Res.* **103**, 241–245
- Montamat, E. E., Moreno, J. & Blanco, A. (1978) *J. Reprod. Fertil.* **53**, 117–123
- Mowbray, J. (1975) *Biochem. J.* **148**, 41–47
- Odessey, R. (1980) *Biochem. J.* **192**, 155–163
- Storey, B. T. & Kayne, F. J. (1977) *Biol. Reprod.* **16**, 549–556
- Sullivan, S. G., Dancis, J. & Cox, R. P. (1976) *Arch. Biochem. Biophys.* **176**, 225–234
- Van Dop, C., Hutson, S. M. & Lardy, H. A. (1977) *J. Biol. Chem.* **252**, 1303–1308
- Van Dop, C., Hutson, S. M. & Lardy, H. A. (1978) *Arch. Biochem. Biophys.* **187**, 235–242