Studies *in vitro* on shuttle systems of mouse spermatozoa

Carlos BURGOS, Carlos E. CORONEL, Nelia M. GEREZ DE BURGOS, Leonor E. ROVAI and Antonio BLANCO

Cátedra de Química Biológica, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, 5000 Córdoba, Argentina

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Observations on systems reconstituted *in vitro* with different starting substrates (2-hydroxy-acids, 2-oxo-acids or leucine) indicate that a branched-chain 2-hydroxy-acid/2-oxo-acid shuttle for the transfer of reducing equivalents from cytosol to mitochondria may be operational in mouse sperm. Evidence is presented suggesting that the 2-oxo-acids produced by intramitochondrial oxidation of 2-hydroxy-acids ingressed from the cytosol can recycle back into the external phase. Observations *in vitro* demonstrate that, in addition to the branched-chain 2-hydroxy-acid/2-oxo-acid shuttle, the malate/aspartate system is also active in mouse sperm. On the contrary, the lactate/pyruvate redox couple does not appear to function as part of a shuttle system in mouse sperm mitochondria. The glycerol 3-phosphate shuttle probably is not functionally significant in mouse spermatozoa, since the activity of the 'soluble' glycerol 3-phosphate dehydrogenase is very low.

It is known that spermatozoa from different species utilize fructose, glucose and lactate as major respiratory substrates (Salisbury & Lodge, 1962; Mann, 1964). Oxidation of these compounds generates NADH in the cytoplasm and makes necessary the existence of mechanisms for the transfer of reducing equivalents to the mitochondria. Among the different shuttle systems so far described, it appears that the malate/aspartate shuttle is functional in spermatozoa (Calvin & Tubbs, 1978).

Studies in this laboratory led to the proposal of an additional system in mouse spermatozoa that utilizes branched-chain 2-oxo-acids as hydrogen acceptors (Blanco *et al.*, 1976). Relevant to this hypothesis was evidence on the subcellular localization (Montamat & Blanco, 1976) and substrate specificity of the lactate dehydrogenase (EC 1.1.1.27) isoenzyme X (LDH-X or 'C₄') specific for spermatozoa and spermatogenic cells (Blanco *et al.*, 1976).

The branched-chain amino acid aminotransferase is present in mouse spermatozoa, with the same subcellular location of LDH-X (Montamat *et al.*, 1978). The 2-oxo-acids produced by transamination of branched-chain amino acids could be reduced by cytoplasmic NADH in a reaction catalysed by the soluble LDH-X. The 2-hydroxy-acids thus formed would penetrate into the mitochondria and be

oxidized by LDH-X, transferring H to the mitochondrial NAD⁺ and then to the respiratory chain. Studies on a reconstituted system *in vitro* supported this hypothesis (Gerez de Burgos *et al.*, 1978).

This paper presents additional observations on the 2-hydroxy-acid/2-oxo-acid shuttle *in vitro* and studies on other shuttle systems in mouse spermatozoa.

Experimental

Materials

Mitochondria. The organelles were obtained from testis of adult albino Swiss mice. The 'heavy' mitochondrial subfraction from adult mouse testis, containing a fairly homogeneous population of the organelles present in the mid-piece of spermatozoa, was obtained as described by Machado de Domenech *et al.* (1972) for rat testicular homogenates. These organelles are designated 'sperm-type' mitochondria.

Enzymes. LDH-X was purified from adult mouse testes by the method of Goldberg (1972). Branched-chain amino acid aminotransferase (leucine aminotransferase; EC 2.6.1.6) was purified from mouse kidney by the procedure described by Aki *et al.* (1968). Units of this enzyme are defined as proposed by those authors.

After purification, the enzymes were dialysed overnight against 0.02 M-Tris/HCl, pH 7.4. The preparation did not show glutamate dehydrogenase activity.

Abbreviation used: LDH-X, lactate dehydrogenase isoenzyme X.

Other enzymes utilized were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Chemicals. All chemicals used were obtained from Sigma (St. Louis, MO, U.S.A.).

Methods

Reconstituted systems in vitro. Malate/aspartate shuttle. The system described by Bremer & Davis (1975) was used.

2-Hydroxy-acid/2-oxo-acid shuttle. Three different systems were utilized. (a) Adapting the conditions indicated by Bremer & Davis (1975) for the malate/aspartate shuttle. The pre-incubation mixture was composed of 3.3 mM-L-glutamate, 2-hydroxy-acid (at concentrations given in the Results and discussion section), 1.7 mM-NAD⁺, 5 units of LDH-X, 3 units of leucine aminotransferase, 25 mM-imidazole/HCl buffer, pH 7.4, and 75 mM-KCl, in a final volume of 3 ml.

(b) With leucine and 2-oxoglutarate as initial substrates. The pre-incubation mixture contained 0.2 mM-L-leucine, 0.5 mM-2-oxoglutarate, 5 units of leucine aminotransferase, 0.775 mM-NADH, 5 units of LDH-X, 25 mM-imidazole/HCl buffer, pH 7.4, and 75 mM-KCl. The final volume was 3 ml.

(c) With 4-methyl-2-oxopentanoate as initial substrate. The pre-incubation mixture contained 4-methyl-2-oxopentanoate 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.

The mixtures were incubated at room temperature (20–23°C) for 20 min and then mitochondria were added. The amount used in each case is indicated in the Results and discussion section. 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. Supernatants were used for NADH assays.

NADH determination. This was performed by the method of Klingenberg (1963).

Determination of 4-methyl-2-oxopentanoate. The incubation mixture was deproteinized with HClO₄. The 2-oxo-acid was determined spectrophotometrically in the neutralized supernatant as oxidation of NADH in the presence of LDH-X.

Glycerol 3-phosphate dehydrogenase. The mitochondrial enzyme was determined by the technique of Singer (1974), and the cytosolic enzyme by the method described by Brooks (1976).

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

Determination of pyruvate and lactate uptake by mitochondria. An adaptation of the procedure used by Halestrap (1978) was used. Mouse 'sperm-type' mitochondria equivalent to 3 mg of total protein were added to 1 ml of incubation medium (75 mM-KCl, 25 mM-imidazole/HCl buffer, pH 7.4, and 2 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) at 37°C. Pyruvate and/or lactate were added up to 0.5 mM concentration. After incubation for 15 min, 0.1 ml of 21% (w/v) HClO₄ was added. Pyruvate and lactate in the medium were determined by enzymic assay (Bergmeyer, 1974) after neutralizing with K₂CO₃.

Control of mitochondrial integrity. The organelles were incubated at 37°C in a mixture containing 0.5 mM-NADH, 25 mM-imidazole/HCl buffer, pH 7.4, and 75 mM-KCl in a final volume of 3 ml during 10 min. Then the remaining NADH was determined. The final NADH/initial NADH ratio $\times 100$ indicates the percentage of integrity. Mitochondria used in all the experiments described gave a value higher than 95%.

Respiratory control index or acceptor control ratio (Chance & Williams, 1956) was also determined. 'Sperm-type' mitochondria gave values varying from 1.8 to 2.0. This is a low value compared with that for liver or heart mitochondria, but it is similar to those obtained by other authors (Keyhani & Storey, 1973) for rabbit sperm mitochondria.

Results and discussion

Activity of the shuttle with different 2-hydroxy-acids

The system reconstituted with 2-hydroxy-acid and L-glutamate as initial substrates is a direct adaptation of that proposed by Bremer & Davis (1975) for the malate/aspartate shuttle. The reaction:

catalysed by LDH-X is responsible for the accumulation of NADH during pre-incubation. On addition of mitochondria, the hydroxy-acid is transported into the organelles, where it is oxidized. This displaces the equilibrium of the reaction to the left, thereby oxidizing preformed extramitochondrial NADH. Studies with this system demonstrated a direct relationship between extramitochondrial NADH oxidized and amount of mitochondria added, up to 10 mg of total protein, or time of incubation up to 20 min. For routine experiments, we selected a time of incubation of 10 min.

The branched-chain 2-hydroxy-acids DL-2-hydroxy-3-methylbutanoate, DL-2-hydroxy-3-methylpentanoate and DL-2-hydroxy-4-methylpentanoate were added to the system at a 25 mM final concentration. This concentration was selected because it assures maximum velocity of LDH-X

Table 1. *Activity of the 2-hydroxy-acid/2-oxo-acid shuttle system with different substrates*
After pre-incubation, 'sperm-type' mitochondria were added in an amount corresponding to 7.6 mg of total protein.

Substrate	Preformed NADH (nmol)	Final NADH (nmol)	Δ NADH (nmol)	Δ NADH (%)	Oxidized NADH (nmol/min per mg of protein)
DL-2-Hydroxy-4-methylpentanoate (25 mM)	209.4	79.9	-129.5	-61.8	1.70
DL-2-Hydroxy-3-methylpentanoate (25 mM)	288.3	126.4	-161.9	-56.1	2.13
DL-2-Hydroxy-3-methylbutanoate (25 mM)	160.3	56.7	-103.6	-64.6	1.36
L-Lactate (25 mM)	115.4	176.8	+61.4	+53.3	—
L-Lactate (12.5 mM)	100.4	247.6	+147.2	+146.8	—
L-Lactate (4.0 mM)	63.5	128.0	+64.5	+101.5	—

(Blanco *et al.*, 1976). L-Lactate was added at three different concentrations: 4.0; 12.5 and 25.0 mM (4.0 mM is close to the K_m value of mouse LDH-X and 25.0 mM to the optimal concentration). Table 1 shows the results of one representative experiment out of five performed in identical conditions. As presented in Table 1, the branched-chain 2-hydroxy-acids were all effective in promoting oxidation of extramitochondrial NADH. On the contrary, there was a substantial increase of NADH in the external phase when lactate was present in the mixture. The increase of NADH was lower with 25.0 than with 12.5 mM-lactate. With 4.0 mM-lactate, addition of 6.0 mM-ADP to the system did not produce oxidation of extramitochondrial NADH.

Controls without substrate and with NADH added to the mixture instead of NAD^+ always showed very poor oxidation of the coenzyme (below 5% of the added amount).

Observed differences in specific activity of the system with the different branched-chain substrates are well correlated with the relative catalytic efficiency of LDH-X with the 2-hydroxy-acids (Blanco *et al.*, 1976).

At variance with the data reported by Storey & Kayne (1977) for rabbit spermatozoa and Calvin & Tubbs (1978) for boar spermatozoa, it appears that the redox couple lactate/pyruvate is not able to transfer H^+ in mouse sperm. This system involves the reaction:

On addition of mitochondria, pyruvate is transported into the organelle by the monocarboxylate carrier (Mowbray, 1975), thereby displacing the equilibrium to the right. As a consequence, more lactate is oxidized and more NADH is generated. The lesser amount of NADH formed when the concentration of added lactate was higher suggests that lactate may compete with pyruvate for the carrier.

Studies carried out to determine pyruvate and lactate uptake by mitochondria demonstrated that consumption of pyruvate in the medium was 47.5

nmol/min per mg of protein, and that the loss of lactate from the medium was markedly lower (2.2 mmol/min per mg of protein). When both substrates were present in the medium at the same concentration (0.5 mM), the amount of pyruvate consumed was the same as with the substrate alone. These results suggest that lactate is not metabolized by mouse sperm mitochondria.

Activity of the system with 4-methyl-2-oxopentanoate and NADH

Since the K_m of LDH-X for 4-methyl-2-oxopentanoate is 0.04 mM (Blanco *et al.*, 1976) and the reaction catalysed by LDH-X is favoured in the direction 2-oxo-acid $\rightarrow$ 2-hydroxy-acid, reconstitution of the system with 2-oxo-acid and NADH permitted the use of much lower concentrations than those utilized for the 2-hydroxy-acids.

Oxidation of extramitochondrial NADH kept a direct relationship with the amount of mitochondria added, or with the time of incubation. Average rate of oxidation of NADH was 3.88 nmol/min per mg of protein.

The effect of 4-methyl-2-oxopentanoate and NADH concentrations on activity of the shuttle is shown in Table 2. Results are from a representative experiment of four performed in identical conditions.

Concentrations of 4-methyl-2-oxopentanoate used were 0.1 mM (experiments A and B), 0.2 mM (experiments C and D) and 0.5 mM (experiment E), and those of NADH were 0.163 mM (experiment A), 0.477 mM (experiments B, C and E) and 0.775 mM (experiment D). Mitochondria were added in an amount corresponding to 2.56 mg of total protein. In all the experiments, the amount of 2-oxo-acid consumed during pre-incubation was identical with that of NADH oxidized, corresponding to the stoichiometry of the reaction. With the lowest concentration of substrate (experiments A and B), the 2-oxo-acid was completely used up. However, after addition of mitochondria there was an additional decrease of external NADH.

In experiments C and D, the amount of 2-oxo-

Table 2. *Effect of 4-methyl-2-oxopentanoate and NADH concentrations on activity of the 2-hydroxy-acid/2-oxo-acid shuttle with mouse 'sperm-type' mitochondria*

Mitochondria were added after pre-incubation in an amount corresponding to 2.56 mg of total protein.

Expt.	Substrates	Amount after			Δ NADH (nmol)	Δ NADH (nmol/min per mg)
		Amount initially added (nmol)	pre-incubation (nmol)	Final amount (nmol)		
A	4-Methyl-2-oxopentanoate	300.0	0	0		
	NADH	489.6	189.6	111.2	-78.4	-3.06
B	4-Methyl-2-oxopentanoate	300.0	0	0		
	NADH	1432.5	1132.5	939.0	-193.5	-7.55
E	4-Methyl-2-oxopentanoate	1500.0	530.0	379.5		
	NADH	1432.5	468.0	312.0	-156.0	-6.09
D	4-Methyl-2-oxopentanoate	600.0	100.0	0		
	NADH	2325.0	1827.5	1578.0	-249.5	-9.74
C	4-Methyl-2-oxopentanoate	600.0	112.0	0		
	NADH	1432.5	940.5	712.5	-228.0	-8.9

acid in the mixture after pre-incubation was 112 and 100 nmol respectively. In both cases, the amount of 2-oxo-acid remaining in the 'cytosolic' phase could not account for the 228 and 249.5 nmol oxidized after addition of the organelles.

These experiments performed by using 2-oxo-acid concentrations lower than those of NADH demonstrate the cyclic nature of the shuttle. The 2-oxo-acids produced intramitochondrially by oxidation of 2-hydroxy-acids ingressed from the 'cytosol' can return to the external phase and promote further oxidation of NADH.

In experiment E, with a high concentration of 2-oxo-acid (0.5 mM), there is a net transfer of reducing equivalents to the mitochondria, but the excess of 2-oxo-acid available in the 'cytosol' does not allow detection of recycling.

There was a direct relationship between concentration of NADH in the original mixture and specific activity of the system. The highest concentration of the 2-oxo-acid produced a reduction of activity, which could be due to substrate inhibition of LDH-X.

Addition of 0.01 mM-rotenone and 0.016 mM-antimycin, two inhibitors of the electron-transport chain, inhibits completely the oxidation of extramitochondrial NADH in the system. This indicates that the transfer of reducing equivalents is linked to the activity of the respiratory chain.

Activity of the system reconstituted with L-leucine and 2-oxoglutarate

The capacity to transfer reducing equivalents was similar whether the system was reconstituted with the amino acid or with the homologue-chain oxo-acid.

Activity of different shuttle systems in sperm mitochondria

The reconstituted malate/aspartate shuttle oxid-

ized extramitochondrial NADH. In the conditions of the system *in vitro*, this shuttle showed activity with mouse sperm mitochondria. Calvin & Tubbs (1978) reported a similar finding with hypo-osmotically treated boar spermatozoa.

Assays of the mitochondrial, FAD-linked, glycerol 3-phosphate dehydrogenase indicated the same activity for 'sperm-type' and liver mitochondria [0.0143 ± 0.0015 unit/mg of protein and 0.015 ± 0.0014 unit/mg of protein (means $\pm$ S.D. of duplicate determinations on three different samples) respectively].

Cytosolic, NAD⁺-linked, glycerol 3-phosphate dehydrogenase was determined in preparations of epididymal spermatozoa as described by Brooks (1976) for rat spermatozoa, except that the washing solution was that proposed by Keyhani & Storey (1973). Mean values of duplicate assays in three different preparations gave an activity of 1.2 ± 0.06 units/mg of protein for liver preparations and 0.03 ± 0.003 unit/mg of protein for mouse spermatozoa. It must be pointed out that, since the enzymes are different, and because of procedural differences, the activity of the 'soluble' enzyme, as indicated here, is not directly comparable with that of the mitochondrial enzyme. The values show that the specific activity of the cytosolic glycerol 3-phosphate dehydrogenase is strikingly lower in spermatozoa than it is in liver, a tissue where the glycerol 3-phosphate shuttle can operate.

The glycerol 3-phosphate shuttle probably is not functionally significant in mouse spermatozoa. A similar observation has been reported by Brooks (1978) for bull and rat spermatozoa.

It is possible that the relative activity of different shuttle systems in spermatozoa varies from species to species. It is also possible that different redox couples (branched-chain 2-hydroxy-acid/2-oxo-acid, lactate/pyruvate) may integrate shuttle systems comprising LDH-X, since substrate specificity and cata-

lytic properties of this enzyme differ among species (C. E. Coronel, C. Burgos, N. M. Gerez de Burgos, L. E. Rovai & A. Blanco, unpublished work). However, the general and unifying character appears to be the dual intracellular location of LDH-X, which enables the enzyme to perform a central role in shuttle systems.

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