Properties of the Testicular Lactate Dehydrogenase Isoenzyme

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1. Studies were carried out with pure lactate dehydrogenase isoenzymes C₄ (LDH isoenzyme X), B₄ (LDH isoenzyme 1) and A₄ (LDH isoenzyme 5) isolated from mouse testis, heart and muscle tissue respectively; with LDH isoenzyme X purified from pigeon testes and with crude lysates of spermatozoa from man, bull and rabbit. 2. LDH isoenzyme X from all species showed greater ability than the other isoenzymes to catalyse the NAD⁺-linked interconversions of 2-oxobutanoate into 2-hydroxybutanoate and of 2-oxopentanoate into 2-hydroxypentanoate. 3. Mouse LDH isoenzyme X presented the broadest spectrum of substrate specificity. It exhibited very similar *K_m* values for a variety of 2-oxo acids: 2-oxopropanoate (pyruvate), 2-oxobutanoate, 2-oxo-3-methylbutanoate, 2-oxopentanoate, 2-oxo-3-methylpentanoate, 2-oxo-4-methylpentanoate, 2-oxohexanoate and 2-oxo-3-phenylpropanoate (phenylpyruvate). The corresponding 2-hydroxy acids were also readily utilized in the reverse reaction. A strong inhibition by substrate and product was demonstrated for the direct reaction. 4. Intracellular distribution of LDH isoenzyme X was investigated in mouse testes. LDH isoenzyme X activity was located in the fraction of 'heavy mitochondria' and in the soluble phase. 5. A possible functional role for LDH isoenzyme X is proposed: the redox couple 2-oxo acid–2-hydroxy acid could integrate a shuttle system transferring reducing equivalents from cytoplasm to mitochondria.

The lactate dehydrogenase (L-lactate–NAD⁺ oxidoreductase, EC 1.1.1.27) isoenzyme, demonstrated in mature testes and spermatozoa of many species and designated isoenzyme X by Blanco & Zinkham (1963), presents a striking cellular specificity. It is found only in cells of the gametogenic progeny and represents 80% or more of the total lactate dehydrogenase activity of spermatozoa (Zinkham *et al.*, 1964).

The existence of this unique molecular form of lactate dehydrogenase in cells of the spermatogenic line suggested that it must be adapted to fulfil very specialized functions.

Comparative studies of catalytic properties have revealed differences between isoenzyme X and the other lactate dehydrogenase isoenzymes common to somatic tissues (Blanco *et al.*, 1975).

The marked sensitivity of LDH (lactate dehydrogenase) isoenzyme X to inhibition by high concentrations of substrate or product when catalysing the forward reaction (pyruvate into lactate) (Battellino *et al.*, 1968; Schatz & Segal, 1969; Battellino & Blanco, 1970*a*) was interpreted as indicating that LDH isoenzyme X is an 'aerobic' isoenzyme, suited to function preferentially in the direction of lactate oxidation (Blanco, 1973). As LDH isoenzyme 1 is

apparently fit for this function, the acquisition of an extra isoenzyme, which involves an additional genetic locus (Blanco *et al.*, 1966), would be difficult to justify if that were the only role of LDH isoenzyme X.

Other peculiar properties of LDH isoenzyme X from different species have been reported by several authors. This isoenzyme showed activity against 2-oxo and 2-hydroxy acids of carbon chains longer than those of pyruvate and lactate (Allen, 1961; Zinkham *et al.*, 1964; Wilkinson & Withycombe, 1965; Blanco *et al.*, 1966; Battellino *et al.*, 1968; Schatz & Segal, 1969; Battellino & Blanco, 1970*b*; Hawtrey & Goldberg, 1970; Kolb *et al.*, 1970; Wong *et al.*, 1971).

These observations appeared without a definite physiological meaning, since the interconversion 2-oxo acid into 2-hydroxy acid, unlike that of pyruvate into lactate, has not been associated with any significant metabolic pathway.

Further studies of activity with a wide variety of substrates and determination of subcellular distribution of the LDH isoenzyme X afforded additional information, which is presented in this paper. A possible functional role for LDH isoenzyme X is proposed.

Experimental

Materials

Enzyme preparations. LDH isoenzyme X from Swiss albino mouse testes was purified by the method proposed by Goldberg (1972). The product obtained had a specific activity of 55.1 units/mg of protein and revealed a single protein fraction when analysed by electrophoresis on polyacrylamide gel.

LDH isoenzyme 1 from mouse heart, LDH isoenzyme 5 from mouse skeletal muscle, and LDH isoenzyme X from pigeon testes (type I; Blanco *et al.*, 1966) were purified by a procedure modified from that of Gerez de Burgos *et al.* (1973). DEAE-cellulose column chromatography was performed by a NaCl stepwise gradient instead of a pH and NaCl gradient. The gradient was established by adding successively the following solutions: (I) 70 ml of 0.01 M-sodium phosphate buffer; (II) 60 ml of 0.01 M-sodium phosphate buffer/1 mM-NaCl; (III) 60 ml of 0.01 M-sodium phosphate/2 mM-NaCl; (IV) 60 ml of 0.01 M-sodium phosphate/5 mM-NaCl; (V) 60 ml of 0.01 M-sodium phosphate/10 mM-NaCl; (VI) 60 ml of 0.01 M-sodium phosphate/25 mM-NaCl; (VII) 60 ml of 0.01 M-sodium phosphate/50 mM-NaCl. All solutions were pH 7.0. The eluate was collected in 5 ml fractions. In pigeon testes preparations, LDH isoenzyme X was obtained in fractions 11–14. LDH isoenzyme 5 from mouse muscle was obtained in tubes 16–19, and LDH isoenzyme 1 from mouse heart, in tubes 72–76. All the enzymes obtained gave single bands of activity when separated on polyacrylamide gel.

LDH isoenzyme X from human, bull and rabbit spermatozoa was studied on whole extracts of washed spermatozoa prepared as indicated by Battellino *et al.* (1968). Zinkham *et al.* (1964) showed that LDH isoenzyme X represents more than 80% of the total lactate dehydrogenase activity in those preparations.

Chemicals. 2-Oxopropanoic (pyruvic) acid, 2-oxobutanoic (α -ketobutyric) acid, 2-oxo-3-methylbutanoic (α -ketoisovaleric) acid, 2-oxopentanoic (α -ketovaleric) acid, 2-oxo-3-methylpentanoic (α -keto- β -methylvaleric) acid, 2-oxo-4-methylpentanoic (α -ketoisocaproic) acid, 2-oxo-3-phenylpropanoic (phenylpyruvic) acid, DL-2-hydroxybutanoic (α -hydroxybutyric) acid, DL-2-hydroxypentanoic (α -hydroxyvaleric) acid, DL-2-hydroxy-3-methylpentanoic (α -hydroxy- β -methylvaleric) acid, and DL-2-hydroxy-5-carboxypentanoic (α -hydroxyglutaric) acid (all as Na⁺ salts); L-2-hydroxypropanoic (L-lactic) acid (as Li⁺ salt); 2-oxohexanoic (α -ketocaproic) acid, 2-oxo-5-carboxypentanoic (α -ketoglutaric) acid, 2-oxo-4-carboxybutanoic (oxaloacetic) acid, DL-2-hydroxy-3-methylbutanoic (α -hydroxyisovaleric) acid, DL-2-hydroxy-4-methylpentanoic (α -hydroxyisocaproic) acid and DL-2-hydroxyhexanoic (α -hydroxycaproic) acid (as free acids) were

obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The free acids were neutralized with NaOH solution before addition to the reagent mixture.

All other chemicals used were of the highest purity commercially available.

Methods

Enzyme assays. Lactate dehydrogenase activity was determined by recording absorbance change at 340 nm produced by the oxidation of NADH or the reduction of NAD⁺. One unit of enzyme is the amount producing the conversion of 1 μ mol of NAD⁺ per min. Assays were performed at 37°C, except when otherwise stated.

Forward reaction. The reagent mixture contained 0.115 mM-NADH, 100 mM-sodium phosphate buffer, pH 7.4, the 2-oxo acid as neutral Na⁺ salt (concentration will be given in each case) and the enzyme preparation, diluted with the phosphate buffer, pH 7.4, to provide a ΔE_{340} of 0.060–0.070 per min when the activity was assayed at 0.2 mM-pyruvate in a 1 cm light-path.

Backward reaction. The reagent mixture contained 0.9 mM-NAD⁺, 10 mM-Tris buffer, pH 9.0, the 2-hydroxy acid as Na⁺ salt (Li⁺ salt in the case of L-lactic acid) and the enzyme preparation, diluted with 0.01 mM-Tris/HCl, pH 7.4, to provide a $\Delta E_{340/1\text{cm}}$ of 0.040–0.050 per min when the activity was assayed with 50 mM-L-lactate.

Cellular fractionation. About 4 g of pooled testes from adult Swiss albino mice were homogenized in 12 ml of 0.32 M-sucrose solution and separated into subcellular fractions by the differential centrifugation method of Machado de Domenech *et al.* (1972). The fraction of 'heavy mitochondria' was further fractionated by means of a sucrose density gradient as indicated by Machado de Domenech *et al.* (1972).

Electrophoresis. Disc electrophoresis was performed on polyacrylamide gels by the technique of Davis (1964).

Results

Substrate specificity

Forward reaction. Mouse LDH isoenzyme X presented significant activity against all the 2-oxo acids assayed except for oxaloacetic acid. Initial velocity plotted against substrate concentration gave the curves illustrated in Fig. 1. The general shape of the curves was very similar for all monocarboxylic acids. Dicarboxylate 2-oxoglutarate gave a sigmoid curve, and there was no activity with oxaloacetate.

K_m and V values, determined from Lineweaver-Burk double-reciprocal plots, are listed in Table 1. Reciprocals of velocities with 0.02, 0.025, 0.03, 0.04 and 0.05 mM substrate concentrations gave linear plots for all monocarboxylic acids, except for 2-oxo-

Fig. 1. Effect of substrate concentration on activity of LDH isoenzyme X from mouse testes (forward reaction)

Initial velocity, expressed as ΔE_{340} per min, is plotted against substrate concentration. Assays were performed with the same amount of enzyme for all substrates. Each point represents average value of five determinations on different preparations of LDH isoenzyme X. Substrate concentrations were 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mm. Substrates assayed were: (a) pyruvate (●), 2-oxobutanoate (○), 2-oxo-3-methylbutanoate (▽), 2-oxopentanoate (▼) and 2-oxoglutarate (◇); (b) pyruvate (●), 2-oxo-3-methylpentanoate (▲), 2-oxo-4-methylpentanoate (△), 2-oxohexanoate (□) and phenylpyruvate (■).

Table 1. K_m and V values for LDH isoenzyme X from mouse testes

In the forward reaction, K_m and V values were measured from double-reciprocal plots (Lineweaver-Burk) of velocities obtained with 0.02, 0.025, 0.03, 0.04 and 0.05 mm concentrations for all substrates, except for 2-oxopentanoate, for which 0.02, 0.025, 0.03 and 0.04 mm were used. In the reverse reaction, for L-lactate, DL-2-hydroxybutanoate and DL-2-hydroxy-3-methylbutanoate, velocities obtained with 2.0, 5.0 and 10.0 mm concentrations were used for the plots. For DL-2-hydroxypentanoate, DL-2-hydroxy-4-methylpentanoate and DL-2-hydroxyhexanoate 2.0, 5.0, 10.0 and 15.0 mm concentrations, and for DL-2-hydroxy-3-methylpentanoate 2.0, 3.125, 6.25 and 12.5 mm concentrations were used for velocity measurements.

Substrate	K_m (mm)	V (units/mg of protein)	V/K_m
Forward reaction (2-oxo acid into 2-hydroxy acid)			
Pyruvate	0.045	55.0	1222
2-Oxobutanoate	0.036	36.0	1000
2-Oxo-3-methylbutanoate	0.044	43.9	1134
2-Oxopentanoate	0.033	49.9	1330
2-Oxo-3-methylpentanoate	0.040	42.5	1062.5
2-Oxo-4-methylpentanoate	0.040	47.0	1175
2-Oxohexanoate	0.038	42.5	1118.4
Phenylpyruvate	0.086	40.8	474.4
Reverse reaction (2-hydroxy acid into 2-oxo acid)			
L-Lactate	3.7	16.5	4.46
DL-2-Hydroxybutanoate	2.2	11.8	5.36
DL-2-Hydroxy-3-methylbutanoate	2.4	14.7	6.02
DL-2-Hydroxypentanoate	2.5	16.0	6.4
DL-2-Hydroxy-3-methylpentanoate	4.0	36.6	9.15
DL-2-Hydroxy-4-methylpentanoate	4.5	26.9	5.97
DL-2-Hydroxyhexanoate	4.7	23.7	5.04

Table 2. Activity of mouse lactate dehydrogenase isoenzymes against different substrates

In the forward reaction, values are the ratio of activities of analogue/pyruvate determined at the same concentration for both substrates. In the reverse reaction, values are the ratio of activities of analogue/L-lactate determined at the same concentration for both substrates.

Analogue	Substrate concn. (mm)	...	LDH isoenzyme 1		LDH isoenzyme 5		LDH isoenzyme X	
			0.05	0.5	0.05	0.5	0.05	0.5
Forward reaction								
2-Oxobutanoate			0.27	0.20	0	0.03	0.73	0.69
2-Oxo-3-methylbutanoate			0	0.02	0	0	1.04	0.93
2-Oxopentanoate			0	0.15	0	0.05	1.23	0.47
2-Oxo-3-methylpentanoate			0	0	0	0	0.89	0.48
2-Oxo-4-methylpentanoate			0	0	0	0	0.96	0.66
2-Oxohexanoate			0	0.10	0	0.03	0.95	0.51
2-Oxoglutarate			0	0	0	0	0.03	0.36
2-Oxo-3-phenylpropanoate			0	0	0	0	0.51	0.50
	Substrate concn. (mm)	...	5.0	50.0	5.0	50.0	5.0	50.0
Reverse action								
DL-2-Hydroxybutanoate			0.05	0.14	0.05	0.03	0.95	0.50
DL-2-Hydroxy-3-methylbutanoate			0.04	0.03	0	0	0.97	0.78
DL-2-Hydroxypentanoate			0.01	0.02	0	0	0.98	0.86
DL-2-Hydroxy-3-methylpentanoate			0	0	0	0	2.41	1.74
DL-2-Hydroxy-4-methylpentanoate			0	0	0	0	1.42	1.50
DL-2-Hydroxyhexanoate			0	0	0	0	1.00	1.31
DL-2-Hydroxy-3-phenylpropanoate			0	0	0	0	0.36	1.11

pentanoate for which activity at 0.05mm was not used. K_m values are of about the same order of magnitude for most of the monocarboxylic acids tested.

Highest activity was recorded with pyruvate as substrate. Maximum velocity with the analogues ranged between 74 and 90% of that obtained with pyruvate. The higher value corresponded to that of 2-oxopentanoate, and the lower one to that of phenylpyruvate.

Maximum activity was attained at the same substrate concentration (0.2mm) for pyruvate, 2-oxobutanoate, 2-oxo-3-methylbutanoate and phenylpyruvate, and at 0.1mm for 2-oxopentanoate, 2-oxo-3-methylpentanoate, 2-oxo-4-methylpentanoate and 2-oxohexanoate.

All 2-oxo monocarboxylic acids presented strong inhibitory action at high concentration. Substrates of longer carbon chain were most effective as inhibitors.

Mouse LDH isoenzyme 1 and isoenzyme 5 reacted very poorly and only with those 2-oxo monocarboxylates of linear carbon chain. Ratios of activities of analogue/pyruvate for isoenzymes 1, 5 and X at 0.05 and 0.5mm substrate concentrations are presented in Table 2.

The LDH isoenzyme X from man, pigeon, rabbit and bull showed higher activity with 2-oxobutanoate and 2-oxopentanoate than LDH isoenzymes 1 and 5 from the same species. The results agreed with those reported by other authors (Wilkinson & Withcombe, 1965; Blanco *et al.*, 1966; Battellino *et al.*,

1968; Kolb *et al.*, 1970). Pigeon, bull, rabbit and human LDH isoenzyme X utilized very poorly the other 2-oxo acids assayed.

Reverse reaction. Fig. 2 shows the curves obtained by plotting initial velocity against 2-hydroxy acid concentration. Highest maximum activity was attained with 2-hydroxy-3-methylpentanoate. Mouse LDH isoenzyme X was also very active against 2-hydroxy-4-methylpentanoate and 2-hydroxyhexanoate.

Inhibition by substrate was very weak or absent up to a 75mm concentration.

There was no activity against 2-hydroxyglutarate, 3-hydroxybutanoate, 4-hydroxybutanoate, glycerol 1-phosphate, malate or glycerol.

Values of K_m and V are listed in Table 1. The lowest K_m value (2.2mm) corresponded to that of 2-hydroxybutanoate; the others ranged between 2.4 and 4.7mm.

Ratios of activities of analogue L-lactate for LDH isoenzymes 1, 5 and X at 5.0 and 50.0mm concentrations of substrate are shown in Table 2.

Inhibition of the forward reaction by 2-hydroxy acids

The reagent mixture was incubated for 10min at 37°C with 5, 10 and 20mm concentrations of L-lactate (Li^+ salt), 2-hydroxybutanoate, 2-hydroxy-3-methylbutanoate or 2-hydroxy-4-methylpentanoate (Na^+ salts) before starting the reaction by addition of the substrate.

Enzymic activity was assayed with 0.05 and 0.1 mM concentrations of pyruvate, 2-oxo-3-methylbutanoate and 2-oxo-4-methylpentanoate.

K_i values were determined from Dixon plots. The results are presented in Table 3. The lowest values were recorded for 2-hydroxybutanoate. In all cases, inhibition was of the competitive type.

Effect of metabolites of the tricarboxylic acid cycle

Battellino & Blanco (1970b) reported a significant inhibition of mouse LDH isoenzyme X by malate and

succinate. Those determinations were conducted at 20°C and apparently, the effect is temperature-dependent.

We have now repeated the study by running parallel assays at 20° and 37°C. Results at 20°C confirmed the finding of inhibition by malate and succinate. On the other hand, the inhibition was very much decreased and became insignificant at 37°C.

It appears, then, that at a more physiological temperature, there is no effect of tricarboxylic acid cycle metabolites on the activity of mouse LDH isoenzyme X.

Effect of temperature on K_m values

Goldberg (1972) and Wheat & Goldberg (1975) reported a striking effect of temperature on K_m values of mouse LDH isoenzyme X. These authors assigned functional significance to the increase of K_m they observed between 32° and 37°C.

We have determined K_m values for pyruvate of LDH isoenzymes 1, 5 and X from mouse tissues at 27°, 30°, 32°, 35° and 37°C (Fig. 3). The curve for LDH isoenzyme X does not show the 'break' at 32°C reported by Wheat & Goldberg (1975). Further, the slope for LDH isoenzyme X is lower than those for isoenzymes 1 and 5, indicating that affinity for substrate of LDH X is less affected by temperature than that of the common isoenzymes.

The conditions of our assays were not exactly the same as those of Goldberg (1972), the most significant difference being the pH of the reagent mixture. Goldberg (1972) used phosphate buffer at pH 7.0, whereas we used the same buffer at pH 7.4. Despite this difference, the results for LDH isoenzymes 1 and 5 were very similar.

Intracellular distribution of LDH isoenzyme X

Machado de Domenech *et al.* (1970, 1972) found that LDH isoenzyme X from rat testes is associated with a special type of mitochondria, which appear first in primary spermatocytes, increase in number in the subsequent cells of the spermatogenic line, and finally form the mitochondrial sheath of the middle piece of spermatozoa.

Fig. 2. Effect of substrate concentration on activity of LDH isoenzyme X from mouse testes (reverse reaction)

Initial velocity, expressed as ΔE_{340} per min, is plotted against substrate concentration. Assays were performed with the same amount of enzyme for all substrates. Each point represents the average value of four determinations on different preparations of LDH isoenzyme X. Substrate concentrations used were 2, 3.125, 6.25, 12.5, 25.0 and 50.0 mM for DL-2-hydroxy-3-methylpentanoate and phenyl-DL-lactate, and 2.0, 5.0, 10.0, 15.0, 25.0, 50.0 and 75.0 mM for all other substrates. Substrates assayed were: L-lactate (●); DL-2-hydroxybutanoate (○); DL-2-hydroxy-3-methylbutanoate (▽); DL-2-hydroxypentanoate (▼); DL-2-hydroxy-3-methylpentanoate (▲); DL-2-hydroxy-4-methylpentanoate (△); DL-2-hydroxyhexanoate (■); phenyl-DL-lactate (□).

Table 3. Inhibition by 2-hydroxy acids of the forward reaction catalysed by LDH isoenzyme X from mouse testes
For details see the text.

Inhibitor	Substrate	K_i (mM)		
		Pyruvate	2-Oxo-3-methylbutanoate	2-Oxo-3-methylpentanoate
L-Lactate		13.5	27.0	28.0
DL-2-Hydroxybutanoate		4.0	4.0	2.75
DL-2-Hydroxy-3-methylbutanoate		13.0	19.0	12.5
DL-2-Hydroxy-3-methylpentanoate		10.7	7.0	10.5

In those determinations, LDH isoenzyme X was detected in the fractions by analysing with starch-gel electrophoresis. Relative activity of isoenzymes was estimated by densitometry of the stained gels. This method is not sensitive enough to reveal some of the isoenzymes present in low concentrations. We have now found that LDH isoenzyme X, present in the soluble phase was missed by that procedure. Despite the low concentration, the total amount of LDH isoenzyme X in the soluble phase is substantial because of the relatively large volume of that fraction. We have arrived at this conclusion by determining

Fig. 3. Effect of temperature on K_m values for pyruvate of lactate dehydrogenase isoenzymes purified from mouse tissues

K_m for pyruvate (mM) is plotted against temperature. Each point represents average value of four K_m determinations on different samples of LDH isoenzyme X (○); LDH isoenzyme 1 (△) and LDH isoenzyme 5 (□).

LDH isoenzyme X activity with 2-oxoglutarate, a substrate shown by Schatz & Segal (1969) to be utilized by rat LDH isoenzyme X.

We have studied the subcellular distribution of LDH isoenzyme X in mouse testes. The peculiar substrate specificity of this isoenzyme allows a selective determination of its activity in the subcellular fractions and afforded a very sensitive method to detect the enzyme. By using 0.1 mM-2-oxo-4-methylpentanoate or 5 mM-2-oxoglutarate, which are not utilized by the common isoenzymes, it was possible to demonstrate that LDH isoenzyme X was, as in rat testes, associated to the so-called 'heavy mitochondria' fraction. An important proportion, which amounted to about 60% of the total LDH isoenzyme X activity, was also demonstrated in the soluble phase. Table 4 presents the results of those determinations.

The pellet of 'heavy mitochondria' from mouse testes was subfractionated in a sucrose density gradient as described by Machado de Domenech *et al.* (1972). The results are comparable with those obtained with rat testes. LDH isoenzyme X appeared in the subfractions containing the peculiar mitochondria characteristic of spermatozoa, as determined by electron microscopy controls.

Liberation of LDH isoenzyme X from mitochondria required treatment with 1% Triton X-100 and freezing-thawing. This suggests that the enzyme must be located in the inner compartments of the organelle. In mitochondria from bull spermatozoa, Baccetti *et al.* (1975) have demonstrated histochemically that lactate dehydrogenase activity is localized in the matrix.

Discussion

The functional role of LDH isoenzyme X has been an intriguing question since its discovery.

Being the major lactate dehydrogenase form in seminal cells, LDH isoenzyme X must be integrated in metabolic pathways that provide energy for

Table 4. Distribution of lactate dehydrogenase activity in subcellular fractions of mouse testes

Activity was determined with 0.2 mM-pyruvate, 0.1 mM-2-oxo-4-methylpentanoate and 5.0 mM-2-oxoglutarate as substrates. Activity with pyruvate indicates total lactate dehydrogenase; that with the analogues, only LDH isoenzyme X. Total activity of the first supernatant after separation of nuclei and debris is taken as 100%. Values are the averages of five fractionations. Activity lost in washing solutions has not been considered in the Table.

	Pyruvate		2-Oxo-4-methylpentanoate		2-Oxoglutarate	
	(Total activity units)	(%)	(Total activity units)	(%)	(Total activity units)	(%)
First supernatant	353.8	100	125.4	100	113.6	100
Heavy mitochondria	63.1	17.8	24.3	19.4	23.4	20.6
Light mitochondria	4.2	1.2	0.6	0.5	0.6	0.5
Microsomal fractions	4.5	1.2	2.0	1.6	1.9	1.7
Soluble phase	171.5	48.5	75.4	60.1	72.3	63.6

spermatozoa motility and survival. But we still do not know which particular metabolic mechanism may require such a specific enzyme.

The studies of catalytic properties have demonstrated some characteristics of LDH isoenzyme X not shared by the isoenzymes common to somatic tissues.

A very general property of LDH isoenzyme X from many species is its ability to catalyse the conversion of 2-oxobutanoate and 2-oxopentanoate into their corresponding 2-hydroxy acids, as well as the opposite reaction.

Mouse LDH isoenzyme X presents an even broader substrate specificity than that of the isoenzyme from other species studied.

Several of the 2-oxo acids readily utilized by mouse LDH isoenzyme X are natural metabolites. Thus 2-oxo-3-methylbutanoate, 2-oxo-4-methylpentanoate and 2-oxo-3-methylpentanoate are produced by transamination (or oxidative deamination) of the branched-chain amino acids valine, leucine and isoleucine respectively. Phenyl pyruvate originates from phenylalanine, and 2-oxobutanoate is an intermediate metabolite of pathways related to the amino acids methionine and threonine.

Oxo acids derived from dicarboxylic amino acids did not appear as viable substrates for mouse LDH isoenzyme X. In the conditions assayed, oxaloacetate was not utilized and 2-oxoglutarate gave significant activity only at very high concentrations. The corresponding hydroxy acids were not utilized.

We have not assayed all the 2-oxo acids that can be formed in animal organisms, but it is possible that other 2-oxo acids derived from amino acids could be used as substrates by LDH isoenzyme X.

The 2-hydroxy derivatives are also produced as natural metabolites. In the case of branched-chain hydroxy acids, their concentration in tissues and body fluids increases in conditions in which the decarboxylation of branched-chain 2-oxo acids is impaired (e.g. maple-syrup urine disease) (Lancaster *et al.*, 1974).

It is not known whether these metabolites occur in spermatozoa, but it has been demonstrated that spermatozoa possess transaminase and oxidative deaminase activities (Salisbury & Lodge, 1962; Mann, 1964) and, of course, the capability to produce 2-oxo acids.

On the other hand, it is well documented that mammalian spermatozoa are capable of active aerobic glycolysis (or fructolysis) and readily oxidize a variety of substrates such as fructose, glucose, lactate, etc. (Salisbury & Lodge, 1962; Mann, 1964).

The results presented here can be analysed in the light of this potential of spermatozoa for aerobic glycolysis.

Cells undergoing aerobic glycolysis or utilizing lactate as external energy source, need a mechanism

for reoxidation of the NADH accumulated in the cytoplasm during these processes. It is known that mitochondrial membranes are not permeable to NADH.

Krebs (1967) has emphasized this fact and pointed out the existence of shuttle systems that transfer reducing equivalents from cytoplasm to mitochondria (the shuttle should work in the opposite direction in gluconeogenic cells).

One of the most important shuttle systems appears to be the redox couple oxaloacetate-malate, interconverted by the NAD⁺-dependent malate dehydrogenases of cytosol and mitochondria.

An additional shuttle could be functioning in spermatozoa. The requirements for the existence of that shuttle can be met. (a) An NAD⁺-dependent enzyme that interconverts 2-oxo acids into 2-hydroxy acids is present in cytosol and in mitochondria. (b) 2-Hydroxy acids and 2-oxo acids can penetrate the mitochondrial membranes. The carrier system for pyruvate described in liver and heart mitochondria is able to transport other monocarboxylates as well (Halestrap, 1975; Mowbray, 1975). If a similar system exists in spermatozoa mitochondria, the monocarboxylates could be actively carried through the internal membrane. However, the presence of a carrier would not be essential, since the monocarboxylates diffuse freely in and out of the inner space of mitochondria (Klingenberg, 1970).

The 2-oxo acids formed by oxidative deamination or transamination of amino acids can act as acceptors of hydrogen equivalents from NADH produced in the cytoplasm during aerobic glycolysis or oxidation of exogenous lactate. The reaction is catalysed by the 'soluble' LDH isoenzyme X.

The 2-hydroxy acids thus formed, penetrate into the mitochondria, where they can transfer the reducing equivalents to NAD⁺ of the mitochondrial pool in the reaction catalysed by mitochondrial LDH isoenzyme X.

The 2-oxo acids may continue their catabolism within the mitochondrion. The multienzyme systems responsible for the oxidative decarboxylation of 2-oxo acids are located in mitochondria (Goedde & Keller, 1967). Alternatively, they could be transaminated into their corresponding amino acids. These processes would maintain a low local concentration of 2-oxo acids and favour the functioning of the shuttle in the proposed direction.

Although the effective operation of the 2-oxo-2-hydroxy monocarboxylate shuttle remains to be demonstrated, the hypothesis advanced here fits the available data and offers an explanation for the possible physiological role of the LDH isoenzyme X.

Another noteworthy finding is the strong inhibitory action of substrate and product on the reduction of 2-oxo monocarboxylates catalysed by LDH isoenzyme X. The 2-hydroxy monocarboxylates act as

inhibitors of the reaction with all other oxo analogues. This could have functional implications; accumulation of 2-oxo acid or 2-hydroxy acid may exert a regulatory action.

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