Kinetic Properties of Rabbit Testicular Lactate Dehydrogenase Isozyme

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SUMMARY

The additional lactate dehydrogenase isozyme from rabbit testis and sperm, lactate dehydrogenase isozyme-1 (B4) from heart, and lactate dehydrogenase isozyme-5 (A4) from liver have been separated and partially purified by preparative starch gel electrophoresis.

Studies performed on these fractions comprised $K_m$, and optimum substrate concentration for pyruvate, $\alpha$-ketobutyrate, and lactate, inhibition by substrate and product, effect of oxamate, oxalate, urea, some citric acid cycle metabolites, pH, and heating.

Results indicate that rabbit testicular lactate dehydrogenase isozyme is a distinct molecular form with peculiar catalytic properties. It showed different behavior against high substrate concentrations whether the direct or the reverse reaction was studied: striking inhibition by pyruvate, no effect with lactate. There is a remarkable correlation between these properties and metabolic requirements of mature spermatozoa.

Lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) from mammalian and avian tissues can be separated into five distinct molecular forms or isozymes.

Although recent observations (1) suggest a more complex structure, it is generally accepted that the five lactate dehydrogenase isozymes are tetramers assembled from two different polypeptide units, designated A and B by Markert (2) (M and H by Cahn et al. (3)). Inasmuch as five is the maximum number of tetramers obtained by combinations of two different units, the finding of a sixth isozyme in mature human testis (designated LDH-X by Blanc0 and Zinkham (4)) and sperm (5) implied the existence of at least one additional polypeptide unit. The synthesis of this additional unit in testis at the time of sexual maturation, and the demonstration that it is the predominant lactate dehydrogenase fraction in mature spermatozoa (6), indicate that this isozyme must accomplish a very specific role.

Some of the unusual catalytic properties of the testicular isozyme initially described by Blanc0 and Zinkham and Zinkham, Blanco, and Clowry (6) have been confirmed by other authors (7, 8). They showed that LDH-X from different species exhibit different behavior when studied with various substrates or coenzyme analogues. These observations raise the question of whether the properties of testicular isozymes from different species correlate with the metabolic requirements of the particular germ cell.

Catalytic properties of LDH-X from several species were studied in order to establish if this type of correlation exists. This paper presents data obtained with lactate dehydrogenase isozymes from rabbit tissues.

EXPERIMENTAL PROCEDURE

Materials

L-lactic acid, $\alpha$-ketobutyrate, NADH, NAD, and oxamic acid were purchased from Sigma; sodium pyruvate, potassium oxalate, and urea, from Merck; hydrolyzed starch for electrophoresis from Connaught.

Adult male rabbits (Flanders giants) were killed by injecting 5 ml of air intravenously. Tissues were removed and used immediately when possible or kept frozen at -20° for a few days until utilized. No differences were observed in results from fresh or frozen tissues. LDH-1 (B4) was obtained from heart; LDH-5 (A4), from liver; and LDH-X, from testis and washed spermatozoa. Rabbit sperm was collected with an artificial vagina and processed immediately.

Methods

Homogenates—Tissues were ground in a Potter-Elvejhem homogenizer with 1 part of distilled water (1:1, w/v) and centrifuged at 20,000 x g for 20 min in a refrigerated centrifuge at 4°. Supernatants were used for electrophoretic studies and enzymatic assays.

Rabbit sperm from single ejaculates or a pool of three or more ejaculates were centrifuged at 3,000 rpm for 15 min. The semi-
inal fluid was discarded and the sperm pellet carefully suspended in 20 volumes of 0.14 M NaCl and centrifuged as previously. The sediment was resuspended in 2 parts of distilled water and frozen and thawed twice. After 20 min of centrifugation at 20,000 × g, the supernatant was saved for studies. Freezing and thawing do not affect the electrophoretic mobility of isozymes. Identical patterns were observed when sonic oscillation was used to disrupt sperm cells.

**Electrophoresis**—Starch gel electrophoresis was performed with the technique proposed by Smithies (9) with the use of the vertical device of Boyer and Hiner (10). For preparative purposes, the toothpiece was modified to get two broad slots that enabled the insertion of up to 1.5 ml of sample for the whole block. Usually, gels were prepared with 12 g of starch per 100 ml of 0.03 M borate buffer, pH 8.6, and 0.3 M borate, pH 8.6, was used in the electrode vessels. Other systems utilized to compare subband patterns and relative mobility of isozymes were: phosphate-citrate, pH 7.0; Tris-HCl, pH 8.0; and the discontinuous system of Poulik (11). A voltage gradient of 6 volts per cm was applied during 14 hours at 4°C.

After electrophoresis, the starch block was sliced, and two narrow strips from both edges were stained for lactate dehydrogenase with the method of Dewey and Conklin (12). When the bands developed, the stained strips were replaced on the rest of the unstained block and the area corresponding to the desired isozyme was cut out.

**Elution**—The cut pieces of starch were frozen at -20°C to disrupt the gel structure. Then, they were thawed at room temperature and eluates obtained by squeezing the spongeliike pieces in a syringe. Studies on the eluates were carried out immediately. Storage of frozen gels or eluates overnight resulted in an important loss of activity.

Purity of isozymes so isolated was controlled by subjecting to electrophoresis on polyacrylamide gel with the technique of Davis (13). After staining for lactate dehydrogenase, a single band of activity was always revealed.

Specific activity of eluates indicated that the purification attained by this method for the different isozymes ranged between 80- and 120-fold.

**Lactate Dehydrogenase Assays**—The methods of Wróblewski and LaDue (14) and of Markert and Ursprung (15) were applied for the direct and reverse reaction, respectively. When α-ketobutyrate was used as substrate, the first method was utilized. Optical density change at 340 mμ was read every minute during 6 min in a Beckman model DU spectrophotometer. Samples were diluted to obtain a ΔA490 from 0.030 to 0.040 per min. Assays were carried out at 20°C.

**Protein Determination**—Total protein concentration was determined by reading optical density at 260 mμ and 280 mμ and applying the following formula (16)

\[
\text{O.D.}_{280} = (1.55 \times \text{O.D.}_{260}) - (0.76 \times \text{O.D.}_{280}) = \text{protein concentration in milligrams per ml.}
\]

**RESULTS**

**Electrophoretic Patterns**

Fig. 1 shows the distribution of lactate dehydrogenase isozymes in several rabbit tissues as revealed by starch gel electrophoresis and histochemical staining. From the variety of tissues studied (heart, liver, kidney, spleen, lung, lens, diaphragm, skeletal muscle from different regions, tongue, esophagus, stomach, small intestine, erythrocytes, ovary, uterus, oviduct, testis, epididymis, sperm, and plasma), only mature testis, epididymis, and sperm exhibited an "extra" lactate dehydrogenase isozyme migrating between LDH-4 and LDH-5. The same results were obtained with the different buffer systems used.

Frequently, subbands were observed in the areas of LDH-2, LDH-3, LDH-4, and LDH-5 from many tissues. Some patterns in Fig. 1 show three subbands in LDH-3 and two in LDH-2 and LDH-4 areas. We could not demonstrate subbands of LDH-5 with the mobility of LDH-X. Although subband patterns changed with the different conditions used and, in some instances, were not shown, LDH-X was always evident with the same relative mobility.

In mature testis, LDH-5 activity is so low that it is not demonstrated by the method employed. LDH-X appears as the only cathodic isozyme. For this reason, contamination of LDH-X eluates by trailing LDH-5 is negligible.

Studies of dissociation and recombination of polypeptide units were repeated in the same conditions previously used (17). The results confirmed former observations. Recombination of subunits from LDH-1 and LDH-X isozymes gave three hybrid forms; one with an electrophoretic mobility slightly higher than that of LDH-2, a second moving in front of LDH-3, and a third intermediate between LDH-3 and LDH-4. No isozyme or subband normally present in rabbit tissues exhibited identical electrophoretic characteristics as the newly formed hybrids.

Recombination of polypeptide chains from mixtures of LDH-1 and LDH-5 always gave origin to the five common isozymic forms in the proportions expected according to the relative contribution of A and B monomers. None of these hybridization
experiments resulted in the production of a band with the electrophoretic mobility of LDH-X.

All of the results that follow represent average values from six to eight determinations performed on different preparations.

**Effect of Substrate Concentration**

**Direct Reaction**—Results obtained with LDH-1 and LDH-5 eluates were similar to those repeatedly shown by other authors with highly purified isozymes. LDH-1, which presented lower $K_m$ and optimum substrate concentration, was inhibited by increasing concentrations of pyruvate. LDH-5 exhibited resistance to inhibition by high substrate concentrations. For LDH-X, $K_m$ and optimum pyruvate concentration were identical with those for LDH-1 (Table I).

The curves shown in Fig. 2 were obtained by plotting initial reaction velocity, expressed as percentage of the maximal activity, against substrate concentration. Curves for LDH-1 and LDH-X are identical in their ascending portion; at higher concentrations of pyruvate, LDH-X presents a more intense substrate inhibition. At 10 mM pyruvate, LDH-5 showed 74% (S.D. ±15.0) of its maximal activity, LDH-1, 52% (±8.0), and LDH-X, only 28% (±7.3). The differences between LDH-1 and LDH-X activities at 2, 5, and 10 mM pyruvate were statistically significant (for all of them, $p < 0.001$).

Total lactate dehydrogenase from crude spermatozoan lysates gave values almost identical with partially purified LDH-X from testis (Fig. 2). It is known that LDH-X in washed rabbit spermatozoa represents about 90% of total lactate dehydrogenase activity, the rest being mainly LDH-1 (6). So, the type of curve obtained is in agreement with what would be expected according to the isoenzymic repertory of sperm.

**Reverse Reaction**—Compared with LDH-5, LDH-1 showed greater affinity for lactate, reached its maximal activity at lower substrate concentration and was inhibited by increasing amounts of lactate (Fig. 3). LDH-5 attained its maximal rate at 150 mM lactate and was not inhibited by 200 mM substrate. For LDH-X, the first portion of the curve was intermediate between those of LDH-1 and LDH-5, but optimum concentration of substrate and resistance to inhibition by high concentrations of lactate were identical with those for LDH-5. Differences in activity between LDH-1 and LDH-X at all the concentrations tested from 10 mM to 200 mM lactate were statistically significant (for all of them, $p < 0.001$).

Total lactate dehydrogenase from crude lysates of spermatozoa gave a curve similar to that of partially purified LDH-X from testis (Fig. 3).

These results demonstrate that, while LDH-1 and LDH-5 exhibit the same type of response to increasing concentrations of substrate in both directions, LDH-X behavior changes whether the direct or reverse reaction is studied. Clausen and Øvissen (7) mention a similar finding with human LDH-X.

To study if those characteristies of LDH-X could be repro

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**Table I**

**Optimum substrate concentrations and $K_m$ of rabbit lactate dehydrogenase isozymes**

For the reactions pyruvate $\rightarrow$ lactate, and $\alpha$-ketobutyrate $\rightarrow\alpha$-hydroxybutyrate, the final concentration of NADH was 0.115 mM and that of phosphate buffer, pH 7.4, 80 mM. For the reaction lactate $\rightarrow$ pyruvate, the final concentration of NAD was 0.9 mM, and that of Tris buffer, 10 mM (0.5 ml of 0.1 M Tris, pH 9.0, for 3 ml of reagent mixture).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>LDH-1</th>
<th>LDH-5</th>
<th>LDH-X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate (mM)</td>
<td>$5 \times 10^{-4}$</td>
<td>$1.5 \times 10^{-4}$</td>
<td>$5 \times 10^{-4}$</td>
</tr>
<tr>
<td>L-Lactate (mM)</td>
<td>$5 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>$\alpha$-Ketobutyrate (mM)</td>
<td>$1 \times 10^{-2}$</td>
<td>$2 \times 10^{-2}$</td>
<td>$2 \times 10^{-2}$</td>
</tr>
<tr>
<td>Pyruvate (mM)</td>
<td>$1.2 \times 10^{-4}$</td>
<td>$2.5 \times 10^{-4}$</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>L-Lactate (mM)</td>
<td>$5.5 \times 10^{-3}$</td>
<td>$2 \times 10^{-3}$</td>
<td>$4.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>$\alpha$-Ketobutyrate (mM)</td>
<td>$1.16 \times 10^{-3}$</td>
<td>$1.6 \times 10^{-3}$</td>
<td>$4.3 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* Obtained from Lineweaver-Burk plots.
plotted against concentration of substrate. Final concentration of NADH was 0.115 mM, that of phosphate buffer, pH 7.4, 80 mM, and that of lactate was 50 mM, 10 mM, and 10 mM.

Determinations with mixtures of LDH-1 and LDH-X were also performed. Ratios LDH-1:LDH-X were 1:1, 3:1, 5:1, 1:3, and 1:5. Again, curves with pyruvate gave the intermediate points expected. Inhibition by substrate increased proportionally to the amount of LDH-X in the preparation.

**Activity against α-Ketobutyrate**—Lactate dehydrogenase is able to utilize as substrates other α-keto acids besides pyruvate (19). However, this ability is not the same for the different isozymes. Previous observations with LDH-X from some species (6, 20) showed a high affinity of this isozyme for α-hydroxybutyrate and α-hydroxyvalerate. In this study, the reactivity of rabbit isozyme with α-ketobutyrate was assayed at the same concentrations used for pyruvate. Optimum concentrations of α-ketobutyrate for the three isozymes were higher than those for pyruvate (Table I). LDH-X presented the lowest apparent $K_m$ and optimum concentration. LDH-1 was also very active against α-ketobutyrate, while LDH-5 reacted poorly with this substrate. The ratio of activity against α-ketobutyrate over activity with pyruvate, both taken at their maximal velocity, was 0.91 for LDH-1, 0.90 for LDH-X, and 0.31 for LDH-5.

Curves obtained by plotting enzymatic activity against α-ketobutyrate concentration were of the sigmoid type (Fig. 4) and did not show inhibition by high concentrations of substrate. LDH-1 and LDH-X gave very similar curves. The sigmoid character of the curve was maintained in the presence of 1.5 mM and 10 mM lactate or 1.66 or 3.22 mM ATP.

**Effect of Product**

Conditions for the study were adapted from those used by Stambaugh and Post (21). The assay mixture was incubated with the product for 15 min before the addition of the substrate. Concentrations of substrate and added product were selected in a range well under the inhibitory levels. In studying the reverse reaction, 10 mM and 25 mM lactate (substrate) were used for LDH-1 and LDH-X, and 50 mM for LDH-5. Results are presented in Fig. 5 by plotting initial reaction velocity, expressed as percentage of the activity without added product, against concentration of added pyruvate. LDH-1 showed a striking inhibition by product, while LDH-5 was scarcely affected. LDH-X exhibits resistance to inhibition by product. The addition of 0.3 mM pyruvate at 25 mM lactate produced a reduction of 30% in activity, while LDH-1 in the same conditions decreased its activity in 62%.

When the direct reaction was studied, 0.1 mM, 0.2 mM, and 0.5 mM were the pyruvate (substrate) concentrations used, and 2 mM, 5 mM, and 10 mM lactate (product) were added. Results were comparable with those presented by Stambaugh and Buckley (22). LDH-X showed a remarkable inhibition by product. At 0.5 mM pyruvate, the activity with 2 mM lactate added was 68% of the initial velocity without product; with 5 mM lactate, it was 58%, and with 10 mM lactate, 53%.

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**Fig. 4.** Effect of substrate concentration upon activity of rabbit LDH-X. ●, pyruvate; ○, α-ketobutyrate. Initial reaction velocity, expressed as percentage of maximal activity, is plotted against concentration of substrate. Final concentration of NADH was 0.115 mM, that of phosphate buffer, pH 7.4, 80 mM, and those of substrate were 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 mM.

**Fig. 5.** Effect of product (pyruvate) upon the reverse reaction catalyzed by rabbit LDH-1 (△, ▽), LDH-5 (▲), and LDH-X (●, ○). Initial reaction velocity, expressed as percentage of the activity without added product, is plotted against concentration of pyruvate. Final concentration of NADH was 0.9 mM, that of Tris, 10 mM, and that of lactate, 50 mM (▲), 10 mM (●, ▽), and 25 mM (○, ▽).

Produced by mixtures of LDH-1 and LDH-5, eluates containing equal enzymatic activities were mixed in varying proportions. LDH-1:LDH-5 ratios were 1:1, 3:1, 5:1, 1:3, and 1:5. These mixtures were assayed at the same substrate concentrations used for the purified isozymes. With pyruvate, the curve for the 1:1 mixture showed all its points intermediate between those for LDH-1 and LDH-5. Remarkably, the curve obtained was an exact average of those for the extreme isozymes alone. When LDH-1 predominated in the mixture (3:1 and 5:1), the points were very close to those for LDH-1 alone. When LDH-5 was more abundant (1:3 and 1:5), the curves were similar to that for pure LDH-5. The same determinations carried out with the mixtures after addition of NaCl up to 0.5 M concentration and freezing and thawing, conditions known to bring about dissociation and recombination of subunits (18), gave results comparable to those for the original mixtures. None of these experiments showed an inhibition by pyruvate in the mixtures higher than that observed for LDH-1.

With lactate as substrate, results with the same mixtures were intermediate between the curves for LDH-1 and LDH-5. There was a striking correlation between affinity for substrate, optimal lactate concentration, and degree of inhibition and those expected according to the relative contribution of each isozyme.

Previous observations with LDH-X from some species (6, 20) showed a high affinity of this isozyme for α-hydroxybutyrate and α-hydroxyvalerate. In this study, the reactivity of rabbit isozyme with α-ketobutyrate was assayed at the same concentrations used for pyruvate. Optimum concentrations of α-ketobutyrate for the three isozymes were higher than those for pyruvate (Table I). LDH-X presented the lowest apparent $K_m$ and optimum concentration. LDH-1 was also very active against α-ketobutyrate, while LDH-5 reacted poorly with this substrate. The ratio of activity against α-ketobutyrate over activity with pyruvate, both taken at their maximal velocity, was 0.91 for LDH-1, 0.90 for LDH-X, and 0.31 for LDH-5.

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It is seen again that the behavior of LDH-X is different according to the direction in which the reaction is assayed.

Dixon plots of results demonstrated that the inhibition by product is of the noncompetitive type. This would indicate that there are separate binding sites for pyruvate and lactate. The hypothesis advanced by Novoa et al. (23) could also be applied to this case. If there is a compulsory sequence of reaction, enzyme-coenzyme-substrate, the configuration of the substrate binding sites will be different for the enzyme-NAD and the enzyme-NADH complexes because NAD and NADH have different steric properties.

**Effect of Metabolites**

DL-Malate, succinate, α-ketoglutarate, oxaloacetate, citrate, L-glutamate, and L-aspartate, diluted in 0.2 M phosphate buffer, pH 7.4, were added to the reagent mixture up to a final concentration of 1.5 mM and incubated 30 min before the substrate was added. The pH deviation in the mixture was always less than 0.1 after the addition of metabolite solutions. None of the compounds used, most of them intermediates of the Krebs cycle or substrates easily incorporated into the cycle, showed any effect upon catalytic activity of the three isozymes tested at the optimum pyruvate concentration. These results do not agree with those originally reported by Fritz (24) showing activation of LDH 5 by many of the citric acid cycle intermediates. The same author observed later that pH variation in the reagent mixture could account for his results (25). Thus, our observations confirm that there is no specific effect of some Krebs cycle metabolites upon rabbit lactate dehydrogenase activity.

**Effect of Inhibitors**

**Oxamic Acid**—Oxamate is a competitive inhibitor of lactate dehydrogenase when the reaction goes to the reduction of pyruvate (23). Its action upon the three isozymes was studied. Oxamic acid was diluted in 0.2 M phosphate buffer, pH 7.4, and added to the reagent mixture 30 min before the substrate. The concentrations used for oxamate were 0.001, 0.01, and 0.1 mM, and for pyruvate, 0.05, 0.1, and 0.5 mM. Dixon plots of results for LDH-X are presented in Fig. 6. $K_i$ of oxamate for LDH-X had a value of $6.4 \times 10^{-4} \text{M}$; for LDH-1, $5.3 \times 10^{-4} \text{M}$.

Action of oxamate upon the reaction $\alpha$-ketobutyrate $\rightarrow$ α-hydroxybutyrate catalyzed by rabbit LDH-X. Reciprocal of the initial reaction velocity is plotted against concentration of oxamate. Final concentration of NADH was 0.115 mM, that of phosphate buffer, pH 7.4, 80 mM, and that of $\alpha$-ketobutyrate, 0.2 (○), 0.5 (●), 1.0 (△), and 2.0 mM (▲).

**Table II**

<table>
<thead>
<tr>
<th></th>
<th>LDH-1</th>
<th>LDH-5</th>
<th>LDH-X</th>
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<tbody>
<tr>
<td>reaction pyruvate $\rightarrow$ lactate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 M urea</td>
<td>102.5 ± 5.9</td>
<td>4.0 ± 6.4</td>
<td>51.0 ± 8.4</td>
</tr>
<tr>
<td>0.2 mM oxalate</td>
<td>37.0 ± 12.2</td>
<td>78.5 ± 8.4</td>
<td>48.0 ± 8.6</td>
</tr>
<tr>
<td>reaction lactate $\rightarrow$ pyruvate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 M urea</td>
<td>67.5 ± 11.2</td>
<td>8.0 ± 3.0</td>
<td>29.0 ± 10.9</td>
</tr>
<tr>
<td>0.2 mM oxalate</td>
<td>74.0 ± 6.8</td>
<td>81.0 ± 11.4</td>
<td>94.0 ± 4.8</td>
</tr>
</tbody>
</table>

for LDH-X. Reciprocal of the initial reaction velocity is plotted against concentration of oxamate. Final concentration of NADH was 0.115 mM, that of phosphate buffer, pH 7.4, 80 mM, and those of pyruvate, 0.05 mM (○), 0.1 mM (●), and 0.5 mM (▲).
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80
60
40
20
c
5
100:
60
40
20
LDH - "X"
LDH - 1
LDH - 5

Fig. 8. Effect of pH upon activity of rabbit lactate dehydrogenase isozymes. LDH-X (top curve), LDH-1 (middle curve), LDH-5 (bottom curve). Initial reaction velocity, expressed as percentage of the maximal activity, is plotted against pH of the reagent mixture before addition of substrate. Points represent results of determinations on six different samples for each isozyme. Final concentration of NADH was 0.115 mM, that of phosphate buffer, 80 mM, and that of pyruvate, 0.5 mM for LDH-1 and LDH-X, and 1 mM for LDH-5.

Effect of pH

Mixtures, containing eluates, NADH, and phosphate buffer with pH ranging from 4.3 to 9.1 and varying in 0.2 from one tube to the next (pH values over 8.0 were obtained by adding 0.2 M NaOH), were incubated for 30 min at 20°. The pH recorded was that of the final mixture. Optimum pH was 7.8 for LDH-1, 7.2 for LDH-5, and 7.35 for LDH-X. Results are presented in Fig. 8 plotting activity against pH. LDH-5 was inactivated by exposure to alkaline pH values, while LDH-1 was less affected (65% of the maximal activity remained after incubation at 8.9). LDH-X showed a narrower zone of activity; it is strikingly inhibited by alkaline media; its activity fell rapidly and only 10% of the maximal activity was present after exposure at pH 8.5.

Effect of Preheating

Eluates were diluted to get the same protein concentration (0.25 mg per ml). To avoid important differences in the ionic media, dilution was performed by adding solution obtained by squeezing portions of starch gel from regions of the same block not contaminated by the run. Samples were heated in a water bath for 15 min. Temperatures ranged from 40 to 65°, with 5° intervals. Immediately after heating, samples were kept on ice until assayed with the optimum concentration of pyruvate. Results are shown in Fig. 9. As many authors have demonstrated before, LDH-5 showed higher thermolability than LDH-1 was the most affected, and LDH-5 showed the lowest inhibition. LDH-X presented an intermediate degree of inactivation (Table II). The reverse reaction is less disturbed by oxalate, specially for LDH-X that is practically not inhibited.

Urea—Eluates were incubated with urea at a 2 M final concentration in the same conditions described for oxalate. In the direct reaction, LDH-1 was not affected, while LDH-5 was almost completely inactivated. LDH-X showed an intermediate sensitivity to urea (Table II). The effect of 2 M urea upon LDH-1 and LDH-X was more intense when the reaction lactate → pyruvate was studied. As for the direct reaction, LDH-5 was markedly inactivated. Differences between LDH-1 and LDH-X sensitivity to urea were statistically significant. It seems that NADH has a greater protecting effect on LDH-1 and LDH-X molecules than the oxidized coenzyme.

Effect of Preheating

Eluates were diluted to get the same protein concentration (0.25 mg per ml). To avoid important differences in the ionic media, dilution was performed by adding solution obtained by squeezing portions of starch gel from regions of the same block not contaminated by the run. Samples were heated in a water bath for 15 min. Temperatures ranged from 40 to 65°, with 5° intervals. Immediately after heating, samples were kept on ice until assayed with the optimum concentration of pyruvate. Results are shown in Fig. 9. As many authors have demonstrated before, LDH-5 showed higher thermolability than LDH-1 was the most affected, and LDH-5 showed the lowest inhibition. LDH-X presented an intermediate degree of inactivation (Table II). The reverse reaction is less disturbed by oxalate, specially for LDH-X that is practically not inhibited.
1. LDH-X exhibited an intermediate stability between the two extreme isozymes.

**DISCUSSION**

The finding of an additional electrophoretic fraction with lactate dehydrogenase activity in testicular homogenates from several species posed a number of questions. One was obviously referred to the catalytic specificity of the enzyme. On account of its high affinity with certain α-hydroxy acids other than lactate, one may ask if it is a lactate dehydrogenase isozyme or an unspecific α-hydroxy acid-NAD oxidoreductase.

As previous studies (17) indicated, LDH-X is composed by subunits that, although different from the A (M) and B (H) monomers from the other isozymes, are able to recombine with them and form functionally active molecules. This fact demonstrates that a high degree of conformational similarity must exist among subunits from the different fractions. Kinetic studies presented support this point of view. Its substrate affinity and catalytic behavior strongly indicate that rabbit LDH-X belongs to the family of lactate dehydrogenase isozymes.

Being a lactate dehydrogenase, another question to solve was whether LDH-X represented an entirely different component of the lactate dehydrogenase isozyme complement of testis or merely a subfraction or a product derived from the isozymes common to all tissues. In a recent paper, Stambaugh and Buckley (22) sustain that LDH-X is a subfraction of LDH-5 and that many other tissues besides testis exhibit that isozyme.

A final answer to this question requires further studies with highly purified material. Until this can be achieved, however, available evidence allows a characterization of the testicular lactate dehydrogenase isozyme.

The existence of subfractions of other isozymes with similar net charge cannot be a proof against the uniqueness of LDH-X. On the other hand, it has been demonstrated that the subfraction pattern changes with electrophoretic conditions (27) while the additional Band X appears as a constant component in the various media assayed. With the use of starch gel electrophoresis and different buffer systems, we could not demonstrate Band X material in any other tissue besides testis, epididymis, and sperm.

As reported by Zinkham et al. (6), the additional isozyme appears in zymograms from rabbit testis about 8 weeks of age, and is related to spermatogenic activity. In mature spermatozoa, LDH-X accounts for 90% of total lactate dehydrogenase activity. A survey on many species revealed that the synthesis of this lactate dehydrogenase isozyme at the time of sexual maturation is a very general biological phenomenon (6) and that LDH-X is a tetramer (Cd) of polypeptides different to the A and B subunits.

Particularly interesting was the evidence on the genetic control of the “extra” polypeptide obtained in a study of several pigeon populations (28). It was clear from those observations that a third genetic locus controls the synthesis of the C subunits in pigeon testis.

More recently, Reesler, Stitzer, and Ackerman (29) have revealed the immunological individuality of LDH-X of human sperm.

Present results afford additional elements to support LDH-X as an independent and distinct isozyme.

Stambaugh and Buckley (22) have found complete identity in catalytic properties between LDH-1 and LDH-X from rabbit and human tissues. Analogous results were reported for human isozymes by Wilkinson and Withycombe (30). Our data do not agree with those findings. Only in a few respects are LDH-1 and LDH-X identical. Kinetics of the three homopolymers LDH-1 (B4), LDH-5 (A4), and LDH-X (C4) reveal that there must be structural differences among them. An important difference is the behavior against increasing concentrations of substrate. (a) LDH-X is significantly more sensitive to high pyruvate concentrations than LDH-1. (b) LDH-X is not affected by high concentrations of lactate to which LDH-1 shows inhibition. Sensitivity to oxalate and urea, and effect of pH and heating suggest also the existence of differences in primary and secondary structures.

From the catalytic properties of LDH-1 and LDH-5, primarily described by Plagemann, Gregory, and Wróblewski (31), other authors (3) have deduced their possible physiological role. Correlation between metabolic characteristics of tissues and lactate dehydrogenase isozyme pattern is well documented (32, 33). The fact that the synthesis of a new isozyme is “switched on” in testis at a precise moment of maturation, and that LDH-X is the most abundant lactate dehydrogenase form in sperm suggest that this fraction represents a highly specialized enzyme, the properties of which should correlate with the metabolic needs of spermatozoa.

It has been demonstrated that rabbit spermatogonia derive energy from glycolysis and that they have predominant aerobic metabolism (34). Furthermore, oxygen tension in the media sperm must traverse to reach the ovum (rabbit uterus and fallopian tube) is high enough to assure a aerobic environment (35). The catalytic behavior of LDH-X is very appropriate for an aerobic type of metabolism. If pyruvate accumulates, lactate dehydrogenase would be inhibited and the substrate driven to final oxidation in the Krebs cycle. On the other hand, it has been demonstrated that important concentrations of lactate are present in sperm fluid (36) and in rabbit uterus after mating (37). The striking resistance to inhibition by high lactate concentrations shown by rabbit LDH-X enables it to oxidize that substrate which can be used then as an external source of energy. None of the other isozymes present such a type of functional duality.

Therefore, we can conclude that rabbit testicular lactate dehydrogenase isozyme (LDH-X) is a unique molecular form with peculiar catalytic properties and able to fulfill requirements that the metabolic conditions of spermatogonia may impose. Thus, LDH-X is probably an important factor related with the vitality and function of the germ cell.

**Acknowledgments**—We wish to thank Drs. Ranwel Caputto, Silvio Barra, and Federico Cumar for reading the manuscript and offering helpful criticisms.

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