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Isolation and Characterization of Bovine Lactate Dehydrogenase X*

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ABSTRACT: Lactate dehydrogenase X (LDH X) from bovine testis has been purified to apparent homogeneity. Its kinetic properties, such as pH optima, temperature stability, and degree of substrate and product inhibition, were found to be different from those of bovine heart and muscle LDH. In addition, ATP had a considerably greater inhibitory effect on LDH X than on LDH isoenzymes 1 and 5. Other metabolites, such as pyrophosphate and citrate, selectively inhibited isoenzyme X, but only at concentrations far above physiologic levels. Since the inhibitory effect of ATP on LDH X occurred at relatively low concentrations, it may be significant in sperm cell metabolism.

In spermatozoa of a number of animal species, most or all of the lactate dehydrogenase (LDH),[1] activity is the property of a protein that is electrophoretically distinct from the LDH fractions in somatic tissues. This enzyme, named LDH X by Blanco and Zinkham (1963), is also present in small quantities in mature testis, but absent from the immature organ (Allen, 1961). It appears to be synthesized first in spermatocytes before the meiotic cell division and subsequently in all stages of sperm development (Goldberg and Hawtrey, 1967). Homogenates from testis also contain the common five LDH isoenzymes which are tetramers of the two polypeptides M or A and H or B (Cahn *et al.*, 1962; Markert, 1962). In an electrophoretic pattern from any tissue, homopolymer H_4 (or isoenzyme 1, which predominates in heart) is the most anodal, homopolymer M_4 (or isoenzyme 5, which predominates in muscle) is the least anodal band, and the three heteropolymers with intermediate mobility represent all possible hybrid forms. LDH X from all species so far studied is found relatively close to the point of application. The bovine enzyme, for instance, has electrophoretic mobility intermediate between isoenzymes 4 and 5. The most obvious questions—namely, whether this enzyme functions as a lactate dehydrogenase and whether its structure is similar to that of the known five isoenzymes—have been the subject of several publications. The specificity of crudely separated LDH X has been studied with different substrates and coenzyme analogs (Blanco and Zinkham, 1963; Zinkman *et al.*, 1963). Subsequent studies have dealt with kinetic properties of partially purified preparations from human sperm and rabbit testis (Stambaugh and Buckley, 1967; Battellino *et al.*, 1968). Very recently, highly purified enzyme was obtained from rat testis (Schatz and Segal, 1969)[2] and studied in respect to several of its properties. The results indicate that NAD^+ and lactate are indeed the preferred substrates of LDH X, although its affinity spectrum is generally broader than that of LDH 1 and LDH 5. LDH X has a molecular weight similar to that of the other isoenzymes (Zinkham *et al.*, 1968; Schatz and Segal, 1969). It also appears to contain four characteristic subunits, since *in vitro* experiments of crude extracts have revealed a tendency for hybridization with subunits M and H (Zinkham *et al.*, 1963; Goldberg, 1965).

In this paper the purification of LDH X from bull testis to apparent homogeneity is described and a number of kinetic properties of the enzyme are compared to the corresponding properties of purified bovine LDH 1 and LDH 5.

Experimental Section

Materials. NAD^+, Na_2NADH, Na_2ATP, NaADP, AMP (muscle adenylic acid), cyclic AMP (free acid), $NaNADP^+$, and Na_4NADPH were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. D,L-Lactic acid (85–90%) was from Merck and Co., Rahway, N. J. Sodium pyruvate and ammonium sul-

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[1] Abbreviation used is: LDH, lactate dehydrogenase.

[2] This paper appeared while our manuscript was in preparation.

fate (special enzyme grade) were from Mann Research Laboratories Inc., New York, N. Y. Sodium α-ketobutyrate, α-ketoisovaleric acid, tris(hydroxymethyl)aminomethane (Sigma 7–9), nitro blue tetrazolium, and phenazine methosulfate were from Sigma Chemical Co., St. Louis, Mo. Acrylamide, *N,N,N′,N′*-tetramethylenediamine, and *N,N′*-methylenebisacrylamide were from Distillation Products, Rochester, N. Y., and ammonium persulfate from E-C Apparatus Corp., Philadelphia, Pa. Coomassie blue, acrylamide monomer (prep-grade = Prep-Cryl), *N,N′*-methylenebisacrylamide (prep-grade = Bis), *N,N,N′,N′*-tetramethylenediamine (prep-grade = Temed), and ammonium persulfate (prep-grade) were obtained from Canal Industrial Corp., Bethesda, Md. Bio-Gel A-0.5m (200–400 mesh) was purchased from Calbiochem, Los Angeles, Calif. Selectacel (DEAE), standard type, and Selectacel (carboxymethyl) standard type, were from Brown Co., New York, N. Y. Lactate dehydrogenase, beef heart (BHLDH), was the product of Worthington Biochemicals Corp., Freehold, N. J. Frozen beef testes were purchased from Morris Rifkin Comp., South St. Paul, Minn.

Spectrophotometric Assay for LDH. The change in absorbance at 340 nm due to the reduction of NAD^+ or the dehydrogenation of NADH was recorded automatically in a Beckman DB spectrophotometer using cuvets of 1.0-cm light path, with the temperature of the cell compartment maintained at 30°. The assay mixture had a volume of 3.0 ml and contained either pyruvate and NADH, or lactate and NAD^+. During the purification procedure, test conditions were 77.5 mM lactate, 5.6 mM NAD^+, and 50 mM pyrophosphate buffer of pH 8.8; the enzyme was added last and the reaction time was 3 min. In the following, this test will be referred to as standard test. For the kinetic studies, 0.1 M Tris buffer rather than 0.05 M pyrophosphate buffer was used and pyruvate or lactate was added last. Measurements were begun 8–10 sec after initiation of the reaction and continued during 30–60 sec. The amount of enzyme was such that for pyruvate reduction the rate of decrease in absorbance did not exceed 0.040/min. Under these conditions the rate was found to be linear both with time and with enzyme concentration for at least 1 or 2 min. For dehydrogenation of lactate, changes in absorbance up to 0.080/min yielded a linear response in regard to time and enzyme concentration during more than 3 min. The molar absorptivity for NADH, $6.22 \times 10^6 \times cm^2 \times mole^{-1}$ (Horecker and Kornberg, 1948), was used to convert the change in absorbance per minute into international units of LDH activity (U).

Protein Determination. Protein was determined according to Lowry *et al.* (1951). During column chromatography the absorption at 280 nm was monitored as a rough indicator of protein concentration.

Analytic Polyacrylamide Gel Electrophoresis. The procedure for separation of LDH isoenzymes described by Dietz and Lubrano (1967) was used with a Beckman paper electrophoresis cell (Durrum type), modified for disc electrophoresis (G. A. Fleisher, unpublished data). A current of 2–3 mA/electrophoresis tube at 360 V was applied for 2.5–3 hr at 5°. The gels were subsequently stained for LDH activity by incubation at 37° in a freshly prepared solution containing 4.5 ml of 1 N sodium lactate, 90.0 mg of NAD^+, 45.0 mg of nitro blue tetrazolium, and 9.0 mg of phenazine methosulfate per 100 ml of 0.1 M Tris buffer (pH 8.3). For visualization of proteins, duplicate gels were fixed for 30 min in 12% trichloroacetic acid and treated at room temperature for 1 hr with coomassie blue solution. The background stain was subsequently eliminated by allowing the gel to stand in 7% acetic acid for 12 hr. This staining method was found to be equally as sensitive as that with Amido Black, and had the advantage of not requiring electrophoretic destaining.

Preparative Polyacrylamide Gel Electrophoresis. A Poly-Prep instrument (Büchler Instruments Inc., Fort Lee, N. J.) was used, consisting of a temperature-regulated electrophoresis apparatus connected to a peristaltic pump, a refrigerated fraction collector, and a regulated direct current power supply to provide a constant current. The cross section of the gel in this apparatus measures 15.8 cm^2. All chemicals used were highly purified, so-called prep-grade. The procedure itself was an adaptation of the two basic procedures recommended by Büchler for the Poly-Prep and by Canalco for the Prep-Disc apparatus. The system consisted of two gels, differing with respect to acrylamide concentration, buffer concentration, and pH in the following way. The upper gel, length 2.3 cm, contained 3.0% Prep-Cryl, 0.1% Bis, 0.01% Temed, and 0.15% ammonium persulfate in 0.003 M Tris-HCl buffer (pH 7.5); the lower gel, length 1.3 cm, contained 4.0% Prep-Cryl, 0.125% Bis, 0.05% Temed, and 0.15% ammonium persulfate in 0.375 M Tris-HCl buffer (pH 8.8). The elution buffer was 0.375 M Tris (pH 8.8) and the electrode buffer was 0.025 M Tris-glycine (pH 8.3). The sample containing 100–120 mg of protein was first dialyzed against the upper gel buffer and then applied to the column in 5% sucrose solution; 0.1 ml of a 0.01% solution of bromophenol blue was added as a marker. The sample volume was not critical and could vary between 10 and 40 ml. A constant current of 35–40 mA was maintained while the voltage gradient varied between 180 and 360 V. Total electrophoresis time was about 5 hr.

Column Chromatography. For gradient elution the Varigrad mixing device (Büchler Instruments Inc.) was used. The gradient produced by varying the number of chambers and the concentration of buffer can be calculated (Peterson and Sober, 1959).

Purification of LDH 1 and LDH 5. Commercial crystalline beef heart LDH was the starting material for the isolation of LDH isoenzyme 1 as described by Pesce *et al.* (1964). The crystalline isoenzyme 1 thus obtained was homogeneous in acrylamide electrophoresis and in the ultracentrifuge and had a specific activity of 98 U/mg of protein (standard test).

For the isolation of LDH isoenzyme 5 from bovine muscle the procedure described by Pesce *et al.* (1964, 1967) was used. The crystalline enzyme was recrystallized twice and appeared then to be homogeneous both in the ultracentrifuge and in acrylamide electrophoresis. The specific activity of this preparation was 203.0 U/mg of protein (standard test).

Hybridization of Isoenzymes X and 1. The technique described by Chilson *et al.* (1964) for the hybridization of isoenzymes 1 and 5 was applied. A mixture of purified isoenzymes X and 1, 1.8 and 3.6 mg, respectively, in 0.5 M potassium phosphate buffer (pH 6.5) was dialyzed at 5° for 12 hr against a 6 M NaCl solution. Subsequently, the NaCl was removed by dialysis against 0.05 M potassium phosphate buffer (pH 6.5).

Results

Purification of LDH X. Total LDH activity in mature bovine testis was 0.1 U/mg of protein. However, most of this activity

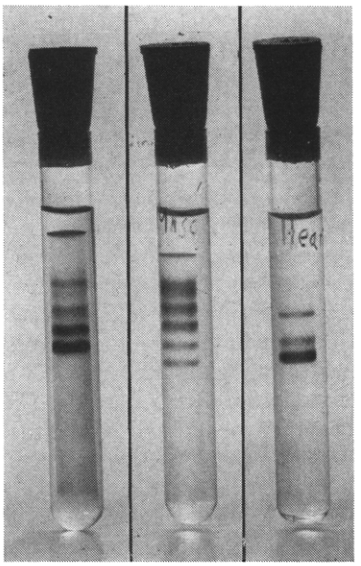

FIGURE 1: Electrophoretic pattern of extracts from bovine testis (left) and bovine muscle (center) and of a commercial preparation of bovine heart LDH (right), all stained for LDH activity.

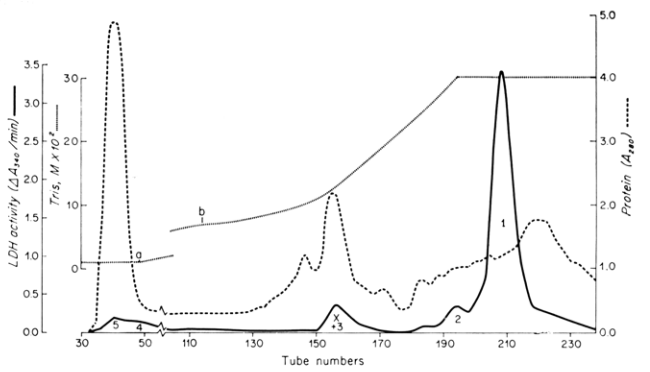

FIGURE 2: Elution profile of LDH isoenzymes from DEAE-cellulose column. Numbers under peaks of LDH activity curve refer to isoenzymes. Small letters refer to changes in eluting buffer: a is the beginning of a linear gradient and b the beginning of an exponential gradient. For details of preparation, see text.

consisted of isoenzymes 1 and 2, with much lesser amounts of isoenzymes 3, 4, 5, and X (Figure 1).

CRUDE EXTRACTION. All subsequent purification and centrifugation steps were done at 5–10°. To 3000 g of fresh or frozen testes, 9000 ml of distilled H_2O was added. The mixture was homogenized in a Waring blender and centrifuged for 45 min at 8000*g*.

pH 5.3 SUPERNATANT. Normal HCl was added dropwise until pH was 5.3. After 30 min at 5° the mixture was centrifuged for 45 min at 11,000*g*.

AMMONIUM SULFATE PRECIPITATION, 0.55–0.70 SATURATION. The pH of the clear supernatant was then adjusted to 7.0 with normal NaOH and solid ammonium sulfate was added to 55% saturation. Per cent saturation was always determined according to Green and Hughes (1955) without applying corrections for the difference in temperature. After standing for 2 hr the precipitate was removed by centrifugation at 11,000*g* for 30 min and discarded. In the supernatant the ammonium sulfate concentration was increased to 70% saturation. Two hours later the mixture was centrifuged (30 min, 11,000*g*). The supernatant of this step was devoid of LDH activity and was discarded. The precipitate contained much of the original LDH X activity, but all the other isoenzymes as well; it was dissolved in a minimal volume of 0.01 M Tris buffer (pH 7.6) and dialyzed against the same buffer.

DEAE-CELLULOSE CHROMATOGRAPHY. The clear, red solution was placed on a DEAE-cellulose column (4.6 × 60 cm) which had previously been equilibrated with 0.01 M Tris buffer (pH 7.6). The elution rate was about 40 drops/min and fractions were collected in 15-ml volumes. LDH activity and absorbance at 280 nm, together with the calculated gradients of the eluent, were plotted against fraction numbers (Figure 2). All fractions containing mixtures of isoenzymes X and 3 (150–170) were pooled. It was noted that their LDH activity in contrast to the activity of the other zones was 40% higher in Tris buffer as compared to pyrophosphate buffer, using otherwise standard assay conditions. This enzyme pool was dialyzed against saturated ammonium sulfate, the resulting precipitate was dissolved in a minimal volume of 0.05 M Tris buffer (pH 7.6) and the solution was dialyzed against the same buffer.

BIO-GEL A COLUMN CHROMATOGRAPHY. The brown-red enzyme solution, in aliquots of 6–8 ml, was placed on a Bio-Gel A column (2.6 × 90 cm) which had been equilibrated with the same 0.05 M Tris buffer (pH 7.6). Elutions were made with the same buffer at a rate of 8 drops/min. The heme protein was eluted more slowly than the LDH, but unfortunately there was considerable overlap. Some of these fractions were applied once more to the same column. The fractions containing the peak of the LDH activity were pooled and concentrated by dialysis against saturated ammonium sulfate.

PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS. After dialysis against 0.003 M Tris buffer (pH 7.5) these preparations were used for preparative electrophoresis. Two well-separated peaks of LDH activity were eluted from the gel column, the first consisting of isoenzyme 3, the second of isoenzyme X. The heme protein preceded slightly the LDH 3 peak, but most of it was eluted together with LDH 3. Figure 3 shows an electrophoretic analysis of the LDH X containing eluates stained for LDH activity and for protein. A single band is seen to be present by both staining techniques. The sedimentation pattern of this preparation is shown in Figure 4. One symmetrical peak was present during the whole run (lower tracing). For comparison, the upper tracing shows a purified preparation of LDH 5 after the first crystallization, demonstrating two peaks, the larger of which coincided with respect to time with the LDH X peak in the lower tracing. The smaller peak could be eliminated by recrystallization. An attempt to crystallize LDH X by adding a small amount of ammonium sulfate to the preparation, kept in 0.05 M phosphate buffer at pH 6.5 and 5°, was unsuccessful. It is possible that the conditions used were not optimal and that a higher pH, approximating the isoelectric point of LDH X, would have been more favorable. The specific activity of such preparations of LDH X, measured with standard test conditions, was 5.0 U/mg of protein. However, when the buffer was changed from 0.05 M pyrophosphate of pH 8.8 to 0.1 M Tris of the same pH, the specific activity increased to 36.0 U/mg of protein, that is, sevenfold. It was then discovered that 0.05 M pyrophosphate strongly inhibits LDH

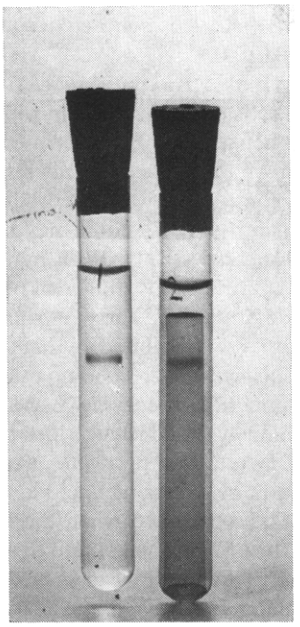

FIGURE 3: Electrophoresis of purified LDH X. Tube 1 was stained for LDH activity and tube 2 for protein.

X but has no effect on the other isoenzymes. The activity of LDH X could be further increased by making the following changes: increase of the lactate concentration from 77.5 to 200 mM, and decrease of the pH of the Tris buffer from 8.8 to 7.5. With these conditions the specific activity was 60.0 U/mg, a 12-fold increase in all. This indicates that the standard test gives far too low values for LDH X, although it is near optimal for the other isoenzymes.

In Table I the total and specific LDH activities for the different purification steps are summarized. Because of the difference in optimal conditions for the isoenzymes, the data obtained with the standard test procedure do not make possible the computation of purification factors.

Comparison of Kinetics of Purified Isoenzymes X, 1, and 5. pH CURVES. All determinations were done in Tris-phosphate buffer containing 0.1 mole/l. each of Tris, phosphate ion, and chloride ion over the pH range of 4.8–10.0 with pH increments of about 0.2. The substrate concentrations used were those

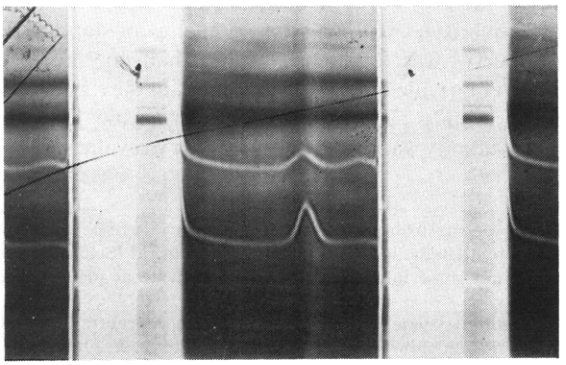

FIGURE 4: Sedimentation patterns of LDH 5 after one crystallization (upper tracing) and of highly purified LDH X (lower tracing). For details of preparations, see text. Picture was taken 15 min after a speed of 59,780 rpm had been reached.

TABLE I: Purification of LDH X.

Steps	Total Protein (mg)	Total LDH[a] (U)	Sp Act. (U/mg of Protein)
Crude extract (from 3000 g of testes)	318,000	29,000	0.09
pH 5.3 supernatant	96,000	18,400	0.13
Ammonium sulfate, 55% saturated supernatant	19,800	8,400	0.43
Ammonium sulfate, 55–70% saturated precipitate	12,600	8,100	0.64
DEAE-cellulose column fractions with isoenzymes X and 3	333	240	0.72
Bio-Gel column, pooled fractions	132	200	1.5
Preparative electrophoresis, pooled fractions with isoenzyme X	3.2	16[b]	5.0[b]

[a] Standard assay conditions. [b] When assayed under optimal conditions—that is, in 0.1 M Tris buffer (pH 7.5) with 3 mM NAD^+ and 200 mM lactate, the values were, respectively, 192 U and 60 U per mg of protein.

which gave maximal velocity at pH 7.5 for each isoenzyme. As is shown in Figure 5 for the pyruvate to lactate conversion, the three isoenzymes had different pH curves. Isoenzyme X had a definite peak at pH 6.6, while isoenzymes 1 and 5 had rather broad optima between pH 6.0 and 7.5. For the reverse reaction, lactate to pyruvate (Figure 6), isoenzyme X had an even narrower peak located between pH 7.4 and 7.6, while

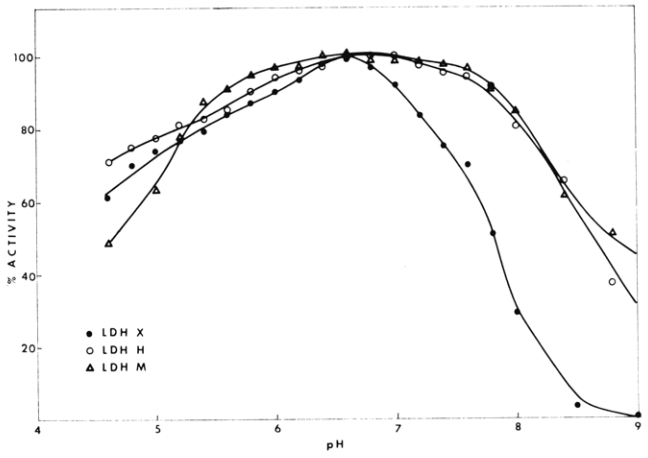

FIGURE 5: Effect of varying pH on activity of LDH X, LDH 1, and LDH 5, with pyruvate and NADH as substrates. Final concentrations: NADH, 0.033 mM; pyruvate, 3.0 mM for LDH X and LDH 5; and 0.6 mM for LDH 1; Tris-phosphate buffer, 0.1 M.

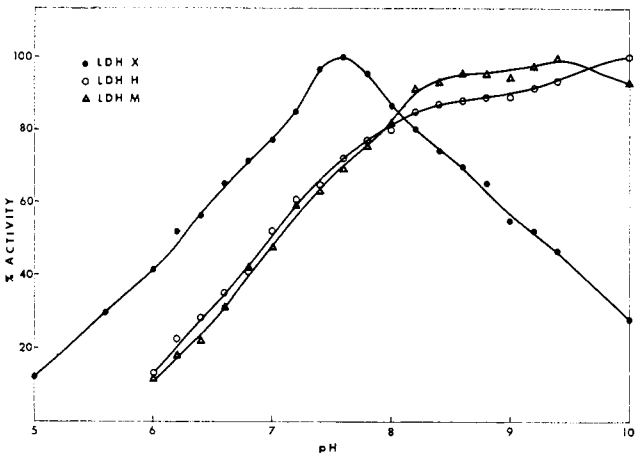

FIGURE 6: Effect of varying pH on activity of LDH X, LDH 1, and LDH 5, with lactate and NAD^+ as substrates. Final concentrations: NAD^+, 3.0 mM; lactate, 200 mM for LDH X and LDH 5, and 40 mM for LDH 1; Tris-phosphate buffer, 0.1 M.

TABLE II: Effect of Varying Substrate Concentrations on Isoenzymes X, 1, and 5 at Different pH Values.[a]

	% Act. of Isoenzyme			
	X		1	5
Substrates				
Pyruvate (mM)	pH 6.6	pH 7.5	pH 7.5	pH 7.5
0.05	27	13	41	21
0.20	55	33	76	39
0.60	85	74	100	77
2.00	100	95	89	96
3.00	95	100	83	100
10.00	59	100	52	88
25.00	49	100	31	45
Lactate (mM)	pH 7.5	pH 8.8	pH 7.5	pH 7.5
0	7	12	39	47
10	38	29	86	35
30	69	46	100	60
100	95	83	88	93
200	100	100	73	100
500	66	100	43	72
α-Ketobutyrate (mM)		pH 7.5	pH 7.5	pH 7.5
0.5		2	14	7
2.0		14	41	17
5.0		44	63	29
10.0		79	69	35
25.0		100	74	42

[a] With pyruvate or lactate, 100% activity is the maximal reaction velocity at the pH indicated. With α-ketobutyrate the activity is expressed as a fraction of the maximal velocity for pyruvate at pH 7.5; NADH, 0.033 mM; NAD^+, 3.0 mM; Tris buffer, 0.1 M.

isoenzymes 1 and 5 showed high activity above pH 8.5 and maxima close to pH 10.0.

EFFECT OF VARYING SUBSTRATE CONCENTRATIONS. Table II summarizes the results of experiments in which the concentration of pyruvate, lactate, or α-ketobutyrate was varied, while the concentration of the respective cosubstrate, NADH or NAD^+, was kept constant. It was noted that, for instance, at pH 7.5 NADPH and $NADP^+$ could not be substituted for NADH and NAD^+, respectively. With pyruvate as the variable substrate, isoenzyme X was studied at pH 6.6 and 7.5, and isoenzymes 1 and 5 at pH 7.5. The data show that at pH 7.5, isoenzymes 1 and 5 were inhibited by high concentration of substrate, while isoenzyme X showed no inhibition. However, at pH 6.6, its optimal pH, isoenzyme X was also inhibited by excess pyruvate, namely, at concentrations above 2.0 mM.[3] In the conversion of lactate into pyruvate at pH 8.8, isoenzyme X gave no inhibition by excess lactate, but at pH 7.5, its optimum, inhibition was observed when the lactate concentration exceeded 200 mM.[4] Isoenzyme 1 and to a lesser degree isoenzyme 5 were both inhibited by excess lactate at pH 7.5. According to Plagemann *et al.* (1960), the degree of substrate inhibition with isoenzymes 1 and 5 is also pH dependent. α-Ketobutyrate, an analog of pyruvate, can be utilized by all three isoenzymes. At pH 7.5, only isoenzyme X reached the same maximal activity with α-ketobutyrate as with pyruvate. Using isoenzyme 1 and isoenzyme 5, the values were lower with α-ketobutyrate, namely, 74 and 42%, respectively, of the values determined with pyruvate. None of the three bovine enzymes showed inhibition by excess α-ketobutyrate. We have also tested α-ketoisovalerate, which reportedly can be utilized by LDH X from the mouse (Allen, 1961), and found that it cannot serve as substrate for any of the three bovine enzymes, at least in concentrations up to 25 mM at pH 7.5.[5]

When NADH was used as a variable substrate at concentrations between 0.003 and 0.100 mM, the saturation curves were found to be sigmoid with all three isoenzymes, most markedly, with isoenzyme X at pH levels above the optimum. With NAD^+, on the other hand, the curves appeared to be hyperbolic. Since such studies involve kinetic measurements at very low substrate concentrations, the relative errors are large and more extensive kinetic studies are needed to establish this difference beyond doubt. In this context it may be mentioned that Anderson and Weber (1965) in their careful studies in the binding of NADH to bovine LDH isoenzymes, in which they used fluorescence techniques, found changes in the apparent affinity of the enzymes for NADH. With isoenzyme 5, but also with the hybrid enzymes, an abrupt change of slope of the titration curves was found when more than one molecule of NADH per enzyme molecule was bound, in the sense of an increase in affinity at this point. For isoenzyme 1, a similar change in affinity was found only at higher pH values.

[3] There appears to be a species difference in this respect since LDH X from rabbit (Battellino *et al.*, 1968) and from rat (Schatz and Segal, 1969) did not show inhibition by excess pyruvate at pH 7.4 and 7.3, respectively.

[4] The rabbit enzyme was tested with lactate concentrations only up to 200 mM and did not show inhibition (Battellino *et al.*, 1968).

[5] According to a recent paper by Schatz and Segal (1969), α-ketoglutarate is utilized by LDH X from the rabbit, with maximal activity equal to 21% of that with pyruvate and a K_m about five times greater than the K_m for pyruvate. We have not tested this substance with our preparation of bovine LDH X.

TABLE III: Substrate Affinity (K_m[a]) for Isoenzymes X, 1, and 5 (in mM).[b]

Variable Substrate	LDH Type X	1	5
Pyruvate	0.40	0.10	0.28
Lactate	14.0	3.0	20.0
α-Ketobutyrate	5.7	2.8	3.6
NADH	0.010	0.010	0.012
NAD	0.13	0.065	0.14

[a] K_m was determined from double-reciprocal plots, except that the values for NADH were estimated from plots of activity *vs.* concentration. [b] Affinity was measured in 0.1 M Tris buffer (pH 7.5). Concentrations of fixed substrates were those corresponding to optimal conditions (see legend of Table IV).

In contrast to the observations with variable concentrations of pyruvate and lactate, we found no inhibition by excess NADH or NAD with any of the three isoenzymes tested up to 0.1 mM NADH and to 5.6 mM NAD. The K_m values for isoenzymes X, 1, and 5—all determined at pH 7.5—are summarized in Table III. In respect to all substrates except NADH, LDH X is more similar to LDH 5 than to LDH 1. The three K_m values for NADH show little differences. For α-ketoglutarate the K_m values were greater than those for pyruvate by more than an order of magnitude. Similar observations were made earlier by Wilkinson and Withycombe (1965) for the human isoenzymes.

PRODUCT INHIBITION. When pyruvate or lactate was used as an inhibitor of the three enzymes rather than as a substrate (Table IV), it was seen that pyruvate had a much greater effect than lactate; the values for isoenzyme X were between those for isoenzymes 1 and 5, but closer to those for isoenzyme 5. These observations are in agreement with those of Battellino *et al.* (1968) for LDH X from the rabbit. NADH as inhibitor affected isoenzymes X and 5 to about the same degree, and more so than isoenzyme 1. NAD^+, on the other hand, inhibited isoenzyme 1 more than isoenzyme 5, while it affected isoenzyme X in a different way, going from weak inhibition at low concentrations (to about 0.2 mM) to strong inhibition at higher concentrations. When our results with NAD^+ were plotted, sigmoid curves were obtained for isoenzymes X and 5, suggesting cooperative effects with respect to coenzyme binding. In contrast, for isoenzyme 1 the curve was hyperbolic.

ATP; Pyrophosphate. The effects of ATP and pyrophosphate on both forward and backward reactions were studied at different pH values and at different concentrations of either NADH or NAD^+. With NADH as the variable substrate, 10 mM pyrophosphate at pH 6.6 had no appreciable effect on LDH X, while 1 mM ATP appeared to cause slight inhibition. When the pH was raised to 7.5, the effects of ATP and pyrophosphate on LDH X became very marked (Table V). It is interesting that the response of this enzyme to the two substances differed markedly; at low concentrations of NADH, ATP inhibited greatly while pyrophosphate had little or no inhibitory effect; at higher concentrations of NADH, the relative activity in the presence of ATP continued to increase, while that in the presence of pyrophosphate reached a plateau, with activity equal to 55% of the maximal activity at pH 7.5 in the absence of inhibitor. Isoenzymes 1 and 5 were not affected by 10 mM pyrophosphate at pH 7.5, irrespective of the substrate (pyruvate or lactate) used. These two isoenzymes did show some inhibition by 3 mM ATP at pH 8.5 and at pH 7.0. These findings with LDH 1 and 5 are in agreement with the observations of Geyer (1967), who furthermore reported that 4 mM ATP had no inhibitory effect on the opposite reaction (lactate to pyruvate) when bovine isoenzymes 1 and 5 were used at pH 7.4. We studied the lactate to pyruvate reaction with isoenzyme X in

TABLE IV: Product Inhibition of Isoenzymes X, 1, and 5.[a]

Product	% Act. of Isoenzyme X	1	5
a Pyruvate (mM)			
0.05	99	60	89
0.10	72	34	83
0.20	63	29	64
0.50	27	15	39
1.00	10	6	29
2.00	5	5	17
5.00	2	4	10
b NADH (mM)			
0.005	84	99	81
0.010	69	96	70
0.020	50	78	54
0.050	30	42	27
0.100	5	18	5
c Lactate (mM)			
1.0	97	96	100
2.0	96	94	100
5.0	95	90	100
10.0	93	84	98
30.0	80	70	96
45.0	75	56	88
100.0	60	36	80
d NAD^+ (mM)			
0.05	98	82	96
0.10	96	76	92
0.20	93	70	82
0.50	78	60	73
1.00	54	50	64
2.00	20	35	52
3.00	10	29	44

[a] Varying amounts of a product were added, while concentration of substrates was held constant corresponding to optimal conditions, namely, for a and b: NAD^+, 3.0 mM, and lactate, 200 mM for isoenzymes X and 5, and 40 mM for isoenzyme 1; for c and d: NADH, 0.033 mM, and pyruvate, 3.0 mM for isoenzymes X and 5 and 0.6 mM for isoenzyme 1. Tris buffer of pH 7.5 was used in all tests.

TABLE V: Effect of ATP on Isoenzymes X, 1, and 5 and of Pyrophosphate on Isoenzyme X with Varying Concentrations of NADH.[a]

	% Act. of Isoenzyme						
	X, pH 7.5			1, pH 8.5		5, pH 8.5	
NADH (mM)	No Addn	ATP (3 mM)	Pyrophosphate (10 mM)	No Addn	ATP (3 mM)	No Addn	ATP (3 mM)
0.01	48	3	40	50	37	46	29
0.02	80	5	43	77	62	70	49
0.03	93	10	47	91	76	83	62
0.05	100	20	53	100	91	95	79
0.10	100	26	53	100	100	100	100

[a] Activity is expressed as percentages of maximum observed velocity, which was determined in the absence of inhibitors at the pH indicated. Final concentrations: pyruvate, 3.0 mM for isoenzymes X and 5, and 0.6 mM for isoenzyme 1; Tris buffer, 0.1 M.

the presence of 1 mM ATP and found, at concentration levels of NAD^+ below its K_m, 20% inhibition at pH 7.5 and 50% at pH 8.8, competitive with respect to NAD^+ (Figure 7). The inhibition by pyrophosphate appeared to be noncompetitive. The lactate to pyruvate reaction in the presence of ATP was not studied with isoenzymes 1 and 5. ADP, AMP, and cyclic AMP at concentrations of 1 mM did not inhibit the three isoenzymes when tested at pH 7.5 with the pyruvate to lactate reaction at low and high levels of substrates and coenzymes.

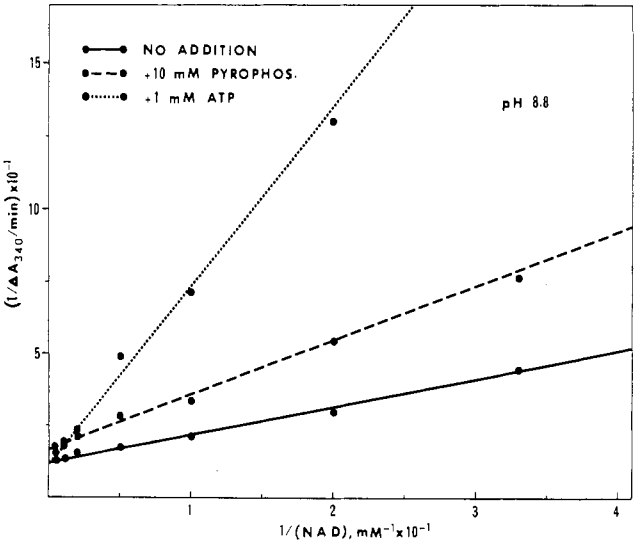

FIGURE 7: Lineweaver–Burk plot of the effect of varying concentrations of NAD^+ on LDH X activity in presence of ATP, of pyrophosphate, and without addition.

TABLE VI: Stability of Isoenzymes X, 1, and 5 at 55°.[a]

	% Act. of Isoenzyme		
Time (min)	X	1	5
3	100	43	30
6	100	41	28
9	100	37	27
12	92	33	25
30	90	26	24

[a] Conditions: 1.0 mg of each enzyme was incubated in 1.0 ml of 0.1 M Tris buffer (pH 7.5).

Citrate; Zinc. When we added 20 mM citrate to isoenzyme X with the substrates NADH and pyruvate in optimal concentrations and the pH at 7.5, there was 53% inhibition. LDH 1 and LDH 5 were not inhibited. At pH 6.6 the inhibition of LDH X was reduced to only 22%. The effect of 0.5 mM zinc chloride on the three enzymes was tested at pH 7.5 with low and high pyruvate concentrations under the following experimental conditions: 5-min preincubation at 30° in the presence of saturating concentrations of NADH. With isoenzyme X the reaction was strongly inhibited at low pyruvate concentrations, but with increasing pyruvate the inhibition decreased and was almost absent at pyruvate concentrations above 10 mM. Under the same conditions, isoenzyme 1 was not affected, while isoenzyme 5 was inhibited by about 80% at low and high pyruvate concentrations up to 25 mM.

Effect of Temperature. Table VI shows comparative studies of temperature stability for the three enzymes, the loss of activity being correlated with exposure time at 55°. It is seen that isoenzyme X was stable under these conditions for the first 9 min, while the activities of isoenzymes 5 and 1 were already reduced after the first 3 min by 70 and 57%, respectively. It is well known that isoenzyme 1 from different species is more resistant to inactivation at elevated temperatures than isoenzyme 5 (Kaplan *et al.*, 1960; Wróblewski and Gregory, 1961; Brand *et al.*, 1962). For the bovine enzymes, Fondy *et al.* (1964) have shown that isoenzyme 5 at 52° lost 80% of its activity after 5 min, while isoenzyme 1 lost only 18%. Our results agree qualitatively with the latter report, but differ quantitatively, mainly with respect to isoenzyme 1.

In a different species, namely the rabbit, the heat stability of isoenzymes X, 1, and 5 has been tested by Stambaugh and Buckley (1967) and by Battellino *et al.* (1968). According to the former report, LDH X and LDH 1 have almost identical stability; according to the latter, LDH X is intermediary between the two other enzymes. Different experimental conditions may account for the discrepancy. At any rate, it can be said that LDH X from rabbit, like that from bull, has greater heat stability than LDH 5.

Hybridization of Isoenzymes X and 1. Subjecting purified LDH X (1.8 mg) and LDH 1 (3.6 mg) to hybridizing conditions resulted in the formation of three new electrophoretic bands with the probable composition X_3H, X_2H_2, and XH_3 (Figure 8). Total LDH activity recovered was 30%. Under similar conditions, also with bovine isoenzymes 1 and 5, the

recovery after hybridization has been reported to be 47% (Chilson *et al.*, 1964).

Discussion

Our investigation has shown the presence of a single fraction of LDH X in bull testis. This is contrary to the finding by Zinkham *et al.* (1964a) who reported three bands X in testis of the same species. They also found two bands X in the guinea pig, but only a single band in man, dog, rabbit, rat, and mouse. Goldberg (1965), on the other hand, noted only one band in bull sperm extract. In our own experience, changing the conditions of electrophoresis from acrylamide gel to starch gel at different pH and to Bio-Gel never yielded more than a single band of LDH X. The discrepancy thus remains unexplained. Zinkham and associates' observation may have been due to a variant of LDH X such as the ones found in pigeons (Zinkham *et al.*, 1964b; Blanco *et al.*, 1964).

In the purification procedure for bovine LDH X, the elimination of heme protein and isoenzyme 3 presented considerable difficulty. Ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration led only to partial separation. Acetone fractionation, which is used in the procedure of Straub (1940) to separate hemoglobin from isoenzyme 1, led to complete inactivation of isoenzyme X. With preparative electrophoresis of partially purified material containing isoenzymes X, 3, and heme protein, the isolation of isoenzyme X was finally possible. The specific activity of purified isoenzyme X, although lower, was of the same order of magnitude as that of crystalline isoenzymes 1 and 5 when each was measured under optimal conditions. Preliminary results on the amino acid composition of LDH X indicated that this enzyme differs considerably in primary structure from both isoenzymes 1 and 5. Nevertheless, hybrid formation between isoenzymes X and 1 occurred under relatively mild conditions.

Among the kinetic characteristics of isoenzyme X, the pH dependence of substrate inhibition—and particularly the absence of inhibition by excess pyruvate at pH 7.5—is noteworthy. Pyruvate inhibition as a regulatory factor may therefore not have a function in the metabolism of bovine sperm cells. On the other hand, ATP at low concentrations of NADH inhibited isoenzyme X much more than isoenzymes 1 or 5, an effect that was also pH dependent. In the physiologic range, the pyruvate to lactate but not the reverse reaction of LDH X was strongly inhibited. These properties may have physiologic significance.

When bovine sperm cells are incubated anaerobically *in vitro* with fructose, their physiologic substrate, or with glucose, the concentration of lactate increases almost linearly with time (Mann, 1964). This means that in the absence of O_2 (and the presence of probably relatively low concentrations of ATP), LDH X efficiently converts pyruvate into lactate, which diffuses out of the cells as soon as it is formed. The cells, however, survive anaerobically in a buffered medium if sufficient hexose is present. Under physiologic conditions, lactate probably is removed by cells of the environment and is thus lost to the sperm cells. When O_2 is added to the medium, CO_2 is formed and the production of lactate decreases. The fate of pyruvate could thus be completely determined by the availability of O_2. The structure of sperm cells with the spacial separation of mitochondria (located in the form of a sheath in the midpiece) and glycolytic enzymes (located in the tail;

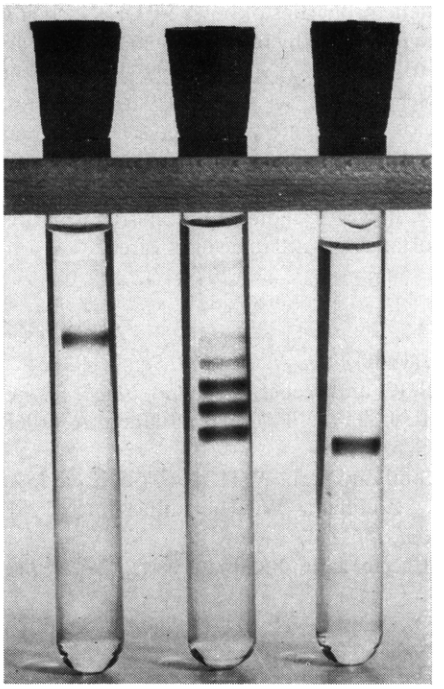

FIGURE 8: Center: electrophoretic pattern of a mixture of LDH X and LDH 1 following *in vitro* hybridization in 6 M NaCl. Left and right: patterns of purified single species, LDH X and LDH 1, respectively, used for experiment.

Nelson, 1953) may, however, be unfavorable for the oxidation of pyruvate, and the formation of lactate could be faster than the transport of pyruvate to the mitochondria. Under these circumstances, the inhibitory effect of ATP on the pyruvate into lactate conversion by LDH X could be beneficial, tending to keep pyruvate within the cells and available for oxidation. Such a mechanism does not seem unreasonable, since the amount of fructose in seminal plasma is limited. In ejaculated bovine semen under anaerobic conditions at 37°, all the fructose would be used up within about 30 min (Mann, 1964). On the other hand, sperm cells are able to use O_2 at very low concentrations, since the rate of O_2 uptake is the same whether the cells are in equilibrium with 1% O_2 or with air.

When sperm cells are incubated aerobically with lactate, which is probably their main substrate in the uterus (Lutwak-Mann, 1962), they survive equally well as with hexose. Apparently LDH X efficiently catalyzes the lactate to pyruvate conversion which, according to our studies, is probably not inhibited by ATP concentrations in the physiologic range.

Thus bovine LDH X seems to be able to function well in both aerobic and anaerobic conditions. Battellino *et al.* (1968) came to the same conclusion from study of the rabbit enzyme, but the mechanisms in the two species may differ. Pyruvate may be preserved for oxidation through the mechanism of excess pyruvate inhibition in the rabbit and through ATP inhibition in the bull. Lactate utilization in rabbit and bull would proceed unhindered, because in both species no inhibition by excess lactate was observed up to 200 mM; in the bull lactate utilization can be enhanced further by the favorable pH optimum for this reaction.

Other kinetic properties of bovine LDH X, such as the susceptibility to inhibition by pyrophosphate, citrate, and zinc, or the ability to utilize pyruvate analogs—and, in rat, α-ketoglutarate (Schatz and Segal, 1969)—as substrates, are not likely to be of physiologic consequence, since the concentration of these substances does not appear to be high enough in sperm cells or seminal plasma. The relative versatility of this enzyme may, however, indicate that more properties of physiologic importance are still to be discovered.

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