Crystalline lactic dehydrogenase of the lens

W. L. Fowlks

A protein with low LDH activity compared to recrystallized muscle LDH and one which appeared to be homogeneous by all available tests for protein homogeneity has been obtained in crystalline form from bovine and rabbit lenses. These lens LDH preparations appeared to be identical immunologically in the other mammalian species examined. The bovine lens LDH had half the molecular weight of LDH isozymes isolated from bovine heart. The LDH activity of bovine lenses in certain chemical tests was found to be both similar and different when compared to bovine heart LDH. This also was the situation with rabbit lens LDH compared with a commercial preparation of rabbit muscle LDH. During a study of the reaction of bovine lens LDH with metallic salts, ZnSO₄ and HgCl₂ in particular, it was noted that the LDH activity became soluble in 2.6M ammonium sulfate, became less stable, and disappeared as a distinct band on disc electrophoresis in acrylamide gel. The chemistry of this transformation in the solubility of LDH suggests that a metal chelate bond was broken by the treatment concomitant with the appearance of a quite unstable low molecular weight fragment with increased LDH activity. Confirmation of this impression awaits success of efforts to isolate such a fragment.

Pirie, van Heyningen and Boag, in a 1953 report concerned with changes in rabbit lenses during formation of cataract induced by x-ray, included results of analysis for lactic acid dehydrogenase (LDH) activity in the homogenates of both normal and cataractous rabbit lenses. In the assay method which they employed, the appearance of DPNH in the incubation mixture was followed by measuring the change in absorbance at 340 mµ as a function of time. This is apparently the earliest report of an actual analysis for LDH activity in the lens from any animal species. The existence of LDH activity in the lens had been suspected for several years prior to this analysis because it has been known since 1941 that excised rabbit and monkey lenses metabolize glucose with the production of lactic acid. The production of lactic acid by any tissue is unlikely in the absence of the lactic dehydrogenase enzyme. Wortman and Becker in 1956 confirmed the presence of LDH activity in rabbit lens homogenates by estimating the pyruvate to lactate reaction, i.e., measuring DPNH utilization. Two years later, Kuhlman and Resnick reported LDH activity in the rat lens. They found LDH activity in the lens capsule with attached epithelium and in the outer cortical layers but found little or no LDH activity in the nucleus.

Recently Fowlks, Gingrich, and Eldridge reported the isolation of a crystalline, apparently homogeneous, protein from the...
lenses of three mammalian species that had LDH activity. Since the isolated protein still contained a major fraction of the total LDH activity of the whole lens homogenate and since efforts to concentrate the LDH activity further had failed, it was assumed that the LDH enzyme of the lens had been isolated, purified, and crystallized.

Maisel, Kerrigan, and Syner have examined the chicken lens for LDH isozymes. They found that the youngest embryonic lens obtainable (4 days of incubation) had a single LDH isozyme line on starch gel electropherograms. The LDH isozyme lines of embryonic chicken lenses had increased to five after 18 days of incubation. During posthatching life, additional lines appeared so that 2-day-old chicks had six LDH isozymes, and 1-year-old chickens had nine LDH isozymes. The embryonic chick lens LDH isozyme pattern resembled the LDH isozyme pattern from chicken breast muscle.

**Isolation of lens LDH activity**

When a homogenate of beef or rabbit lenses was processed by the ammonium sulfate fractionation procedure used by Nielands to obtain crystalline LDH from beef heart, that fraction which would have had LDH activity if beef heart had been used did not have detectable LDH activity when assayed for LDH activity. Assay of the supernatant solution from which LDH activity should have precipitated revealed that most of the LDH activity of the original lens homogenate was still in solution. Upon further careful investigation it was found that, unlike beef heart LDH or rabbit muscle LDH which precipitate quantitatively from 1.9M and 2.1M ammonium sulfate solutions, respectively, the LDH activity from rabbit or beef lenses did not begin to precipitate from solution until the ammonium sulfate concentration was increased to 2.4M. Lens LDH activity precipitated quantitatively only from 2.6M (63 per cent saturated) ammonium sulfate solution when kept at 4° to 10° C. Details of the method for isolation of a crystalline protein which contains as much as 80 per cent of the total LDH activity of the original lens homogenate have been reported fully elsewhere. By use of this isolation procedure, crystalline proteins with LDH activity have been obtained from rabbit, beef, dog, cat, and human lenses.

The supernatant solution obtained by centrifuging a lens homogenate which had ammonium sulfate added to a concentration of 1.7M gave a protein precipitate if the ammonium sulfate concentration was further increased to 2.6M. This 1.7 to 2.6M precipitated protein fraction contained appreciable aldolase activity which dissolved incompletely in 2.0M ammonium sulfate solution. Aldolase activity was reduced further upon each recrystallization from 2.45 to 2.5M ammonium sulfate solution. No aldolase activity was detectable in the crystalline protein obtained after three or four recrystallizations.

The crude LDH fraction, i.e., the protein which precipitated when the ammonium sulfate concentration was increased from 1.7M to 2.6M, contained at least four different proteins separable by paper electrophoresis. The number of proteins in the LDH fraction was reduced to two after one recrystallization and to only one, detectable on paper electropherograms, after four or five careful recrystallizations. With each of the first three or four recrystallizations the specific activity of the preparation increased. In our hands, additional recrystallizations never increased the specific activity further. This result suggested that the crystalline protein with LDH activity isolated from the lens was homogeneous. Further evidence of protein homogeneity by the phase rule criterion as suggested by Northrop was provided by the observation that there was no significant difference between the specific activity of the residue and the supernatant solution when a 5 times recrystallized bovine lens LDH preparation was allowed to equilibrate with sufficient saturated NaCl solution or 2.0M MgSO₄ solution to just dissolve almost all of the aliquot of protein taken and when one
tenth that amount of solution was added to another aliquot of protein.

Chromatography of once recrystallized bovine lens LDH protein fraction on a column of diethylaminoethyl cellulose (DEAE cellulose) gave two peaks of LDH activity in effluent fractions, with the use of a modification of the method reported by Moore and Wortman, who obtained three LDH fractions from rabbit cornea. The second peak of lens protein had constant specific activity for LDH in all fractions. However, a rerun on the same DEAE cellulose column of the protein isolated from either peak again gave two peaks at elution volumes identical to those obtained from the first chromatogram. Protein isolated by ammonium sulfate precipitation from the fractions of the second peak of DEAE cellulose column chromatograms appeared to be homogeneous in the analytical ultracentrifuge, both in sedimentation velocity studies and in sedimentation equilibrium studies. With the assumption that the value for the partial specific volume of the crystalline lens protein with LDH activity fell within the range of partial specific volumes of those proteins for which this parameter has been measured and with the data from the ultracentrifuge, it was possible to calculate a probable molecular weight of bovine lens LDH. The molecular weight calculated was in the range of 60 to 75 thousand which is approximately half of the generally accepted value of 135,000 for the molecular weight of the LDH isozymes from a number of mammalian sources including beef heart and rabbit muscle. It should be noted at this point that finding a lower molecular weight for the crystalline lens proteins with LDH activity is consistent with the finding that a higher concentration of ammonium sulfate is needed in solution to make lens LDH activity insoluble.

When the LDH-rich protein precipitated during the ammonium sulfate fractionation procedure (1.7 to 2.6M precipitate) of decapsulated bovine lenses was submitted to acrylamide gel electrophoresis, four or five protein bands (amido black B stained) and only one LDH isozyme (nitroBT stained) were typically obtained (Fig. 1). Additional LDH bands with lower electrophoretic mobility were sometimes seen if the lens homogenate was not processed and the electrophoresis procedure completed the same day.

Agar gel microelectrophoresis and disc electrophoresis in acrylamide gel of bovine
lens protein from the second peak of DEAE cellulose chromatography or a 5 times recrystallized preparation with LDH activity revealed one protein line and one line with LDH activity. The protein stain and LDH activity stain appear to occupy the same position on the electropherograms.

A fifth criterion of protein homogeneity for the crystalline lens protein with LDH activity is provided by the finding that the lens LDH protein acts like a single antigen in immunoelectrophoresis studies with whole lens antibodies.5

Despite all the evidence for homogeneity of the protein fraction with LDH activity isolated from the bovine lens, the disturbing fact remains that the best preparations of bovine lens LDH we have been able to obtain still have only approximately one five hundredth of the specific activity of a 2 times recrystallized beef heart LDH obtained from commercial sources. This estimate is based upon assays for LDH activity and for protein as used by us on LDH preparations from both sources. The protein fraction with LDH activity isolated from rabbit, dog, or cat lenses appears to have a specific activity identical to that of the bovine lens preparation. The bovine, rabbit, and dog lens LDH appears to have identical antigens by the Ouchterlony technique with antiserum against dog lens prepared in rabbits.

**Heat inactivation of bovine lens LDH**

Kaplan and Ciotti10 have reported on the heat inactivation of bovine heart muscle LDH. Although details of the experimental procedure used were not included or cited in their report, it was indicated that they had heated the LDH preparation for 15 minutes at the indicated temperature before assay for residual activity. Aliquots of bovine lens LDH, a commercial preparation of bovine heart LDH, and a commercial preparation of rabbit muscle LDH were each dissolved in Tris-acetate buffer, pH 7.35, so that each mixture had about the same LDH activity. Aliquots (0.2 mL) of these solutions were heated 15 minutes in a water bath set at a predetermined temperature (±0.02°C). The enzyme solution was then quickly assayed for LDH activity. The enzyme activity of the bovine lens preparation decreased slightly at temperatures between 20° and 50° C. and sharply thereafter to 60°, at which temperature all the enzyme activity disappeared, presumably because the protein was heat denatured, i.e., a precipitate of coagulated protein formed. In contrast, rabbit muscle LDH still had 22 per cent of its original activity and bovine heart LDH had 63 per cent of its original activity after exposure to 60° for 15 minutes. The aliquots of the latter two LDH preparations which were heated to 65° C. had no activity. It appears from these data that bovine lens LDH was more heat sensitive than bovine heart LDH but less heat sensitive at temperatures below the coagulation temperature than the rabbit muscle LDH preparation used (Table I). The effect on these data of pH and buffer composition has not been studied, but apparently these variables have an effect, since Kaplan and Ciotti10 found no inactivation of bovine heart LDH at temperatures below 60° C.

**Chemical properties of lens LDH**

It has been reported previously that:

1. Lens LDH, particularly of rabbit or bovine origin, is specific for L (+) lactic acid and for DPN-DPNH. TPNH was not reduced in the presence of pyruvate.

2. Lens LDH catalyses both the pyruvate to lactate, and the reverse reaction. The ratio of the forward to reverse reaction for beef lens LDH was 3.0. The maximum rate of the reverse reaction was found to occur at about pH 9.0 for both rabbit and bovine lens LDH.

3. The enzyme activity was unaltered by a 30 minute preincubation with the metal complexing agents: cyanide, 8-hydroxyquinoline, diethyldithiocarbamate, 1,10-phenanthrolone, or ethylenediamine tetracetic acid (EDTA), although the enzyme...
Table I

<table>
<thead>
<tr>
<th>Source of LDH</th>
<th>Temperature*</th>
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<tbody>
<tr>
<td></td>
<td>50° C. (%)</td>
</tr>
<tr>
<td>Bovine heart (Sigma)</td>
<td>92</td>
</tr>
<tr>
<td>Rabbit muscle (Sigma)</td>
<td>54</td>
</tr>
<tr>
<td>Bovine lens</td>
<td>93</td>
</tr>
</tbody>
</table>

*Temperature to which enzyme in Tris-acetate buffer (0.1M, pH 7.35) was heated for 15 minutes just prior to assay at 21° to 22° C. Reported as per cent of original activity.

...was slowly inactivated by contact with EDTA for periods longer than 16 hours.

4. Neither the sulfhydryl reagent, N-ethylmaleimide, nor lead salts inactivated the enzyme during one hour's incubation, but lens LDH activity was slowly inhibited by low concentrations of HgCl₂, an inhibition that was completely reversed by complexing the mercury with diethylthiocarbamate just prior to assay.

5. The LDH activity was inhibited completely in borate buffer. Oxamate inhibited lens LDH competitively. Half maximal velocity was obtained with $2.5 \times 10^{-3}$ M oxamate.

6. The preparation had no detectable malic acid dehydrogenase activity.

In further studies of the chemical properties of lens LDH activity it has been found that p-chloromercuribenzoate ($10^{-4}$ M) completely inactivated the enzyme rapidly and irreversibly; unlike bovine heart LDH, neither mercury complexing agents nor sulfhydryl reagents such as glutathione or cysteine restored the lost enzymic activity. Thioacetamide ($10^{-4}$ M) also completely and irreversibly destroyed enzyme activity of lens LDH preparations.

Iodoacetate and iodoacetamide did not inactivate bovine lens LDH when incubated at room temperature in Tris-chloride buffer at pH 7.4 for 6 hours.

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The relative reaction rates with deamino-DPN (Pabst) compared to DPN (Sigma) with the use of both bovine lens and bovine heart LDH are given in Table II. Rabbit muscle LDH is included for comparison. Kaplan and Ciotti¹⁰ have reported a ratio of the rate with deamino-DPN:DPN for bovine heart LDH as 1.05. The corresponding value by the method we used was 1.00, while bovine lens LDH gave a ratio of 0.90 and rabbit lens LDH 0.76, compared to 0.58 for a commercial rabbit muscle preparation. In these studies conditions were adjusted so that the pH at the end of the assay was 9.2 to 9.3. It was found that if the assay was run at pH 9.9, for example, the deamino-DPN:DPN ratio was 0.78 with bovine heart LDH.

Winer and Schwert¹¹ reported that pyrophosphate buffer reduced the initial reaction rate of bovine heart LDH to 60 per cent compared to Tris-chloride buffer at the same pH. In our hands, sodium pyrophosphate buffer (0.1M, pH 9.0) reduced the lactate to pyruvate initial reaction rate by 15 per cent, in comparison to the rate in glycine-NaOH buffer (0.1M, pH 9.0) with the use of bovine heart LDH, but no reduction in the initial reaction rate was found in a similar experiment with bovine lens LDH.

Reactions with Metallic Salts

The crystallization of rat tumor (Jensen sarcoma) and rat skeletal muscle LDH in the presence of mercuric sulfate was re-
ported in 1943 by Kubowitz and Ott.\textsuperscript{12} They regained enzyme activity from the inactive mercury salt of LDH by dialysis against solutions that contained HCN (0.01M), or by adding cysteine to the assay mixture. We have reported previously\textsuperscript{5} that bovine lens LDH was slowly inactivated after the addition of small quantities of mercuric chloride solution. Full activity was restored if the assay mixture contained diethyldithiocarbamate (0.5 mg. per milliliter).

The addition of lead acetate (final concentration $10^{-5}$M) to bovine lens LDH in Tris-acetate buffer, pH 7.3 to 7.4, did not inactivate the LDH nor did lead nitrate in Tris-nitrate buffer under similar experimental conditions. When these experiments were performed with impure lens LDH preparations a precipitate usually appeared during the first day after mixing. However, there was no loss of LDH activity in the precipitate nor was the LDH activity lost or noticeably altered after such mixtures were kept as long as 36 days at 4°C to 10°C. A number of other heavy metal salts, CoCl$_2$, Fe(NH$_4$)$_2$(SO$_4$)$_2$, MnCl$_2$, NiCl$_2$, and SrCl$_2$, were tested individually in similar experiments. There was no measurable inactivation of bovine lens LDH during 20 hours' incubation at 4°C to 10°C, with any of these heavy metal salts at $10^{-5}$M, but AgNO$_3$ at $10^{-5}$M in Tris-nitrate buffer (0.1M, pH 7.4) immediately inactivated the enzyme. Cysteine or other silver complexing agents did not restore LDH activity to the Ag$^+$ inactivated enzyme. The addition of CuSO$_4$, $10^{-4}$M, in a similar experiment resulted in 50 per cent loss of activity in 60 minutes and complete and irreversible loss in 6 hours. Higher concentrations of cupric salts resulted in immediate and complete inactivation of the bovine lens LDH. In each case there was protein precipitation beginning immediately after addition of the copper or silver salt solution. Cadmium chloride at $10^{-5}$M did not inactivate bovine lens LDH in 60 minutes. However, there was complete loss of enzyme activity when $10^{-3}$M CdCl$_2$ in Tris-chloride buffer (0.1M, pH 7.3) was incubated at 4°C to 10°C, for 24 hours. Diethyldithiocarbamate (0.5 mg. per milliliter) did not restore activity during incubation for 20 minutes when added to

\begin{figure}
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\includegraphics[width=\textwidth]{Fig2.png}
\caption{Comparison of the extent of inactivation of bovine lens LDH after 24 and 48 hours' incubation, at 4°C to 10°C, in 2.0M NaCl containing ZnSO$_4$ at $10^{-5}$ to $10^{-3}$M and 2.0M (NH$_4$)$_2$SO$_4$ containing $10^{-5}$M ZnSO$_4$. The enzyme concentration in each solution was initially the same, i.e., enough to give a change in absorbance of about 0.100 per minute.}
\end{figure}
the buffer-washed precipitate that formed on treatment with CdCl₂ or to the supernatant solution obtained by centrifugation of the incubation mixture.

Zinc sulfate at 10⁻⁴ M will facilitate crystallization of lens LDH. It was noted, however, that crystalline preparations of lens LDH stored in 2.6M ammonium sulfate with ZnSO₄ (10⁻⁶ or 10⁻⁵ M) lost enzyme activity during a single month of storage. Part of the activity became soluble in the 2.6M ammonium sulfate but the soluble activity was quickly lost. In contrast, lens LDH preparations from zinc-free solutions in 2.6M ammonium sulfate have been refrigerated for 4 years with little or no loss of LDH activity. Higher concentrations of Zn ion (10⁻⁴ or 10⁻³ M) caused loss of lens LDH activity more rapidly if there was sufficient chloride ion present. The data for 2.0M NaCl compared to 2.0M (NH₄)₂SO₄ is shown in Fig. 2. Diethyl-dithiocarbamate or 1,10-phenanthroline did not restore LDH activity lost when Zn ion was present in the solution.

In experiments with mercuric salts it appeared that mercuric ion reacted with LDH more rapidly and differently than did the zinc. A titration curve of bovine lens LDH in 0.1M Tris-acetate buffer, pH 7.4, with HgCl₂ solution is shown in Fig. 3. Ten minutes at room temperature was allowed after each addition of HgCl₂ solution before the enzyme was assayed by the usual method. Much larger concentrations of HgCl₂ resulted in precipitation of protein and enzymic activity. Full enzymic activity was obtained if diethyl-dithiocarbamate (0.5 mg. per milliliter) was present in the assay mixture even with 10⁻³ M HgCl₂. The reaction of bovine lens LDH with mercuric chloride was not a very rapid reaction, as could be shown by adding all the HgCl₂ solution sufficient to completely inactivate all the LDH activity present in 24 hours and then taking aliquots of the LDH-HgCl₂ mixture for assay by the standard method immediately after mixing and at predetermined times thereafter. In such experiments it takes approximately 30 minutes to reduce the activity to 50 per cent and 16 to 24 hours to reduce the activity to zero. Again full enzymic activity was restored in these mixtures even after 96 hours in the refrigerator if diethyl-dithiocarbamate was present in the assay mixture.

Such LDH-HgCl₂ solutions after 6 hours' incubation at room temperature at 21° to 22° C. gave a crystalline precipitate with all the LDH activity, if the solution was made 2.6M ammonium sulfate by addition of sufficient solid ammonium sulfate, or saturated solution of ammonium sulfate. After 12 to 18 additional hours in the refrigeration mixture, a crystalline precipitate was formed.
erator only part of the original LDH activity was recoverable by making the solution 2.6M in ammonium sulfate, although at this time the solution usually had 1.2 to 1.3 times as much LDH activity per milliliter as was present originally, if assayed with Hg complexing agent present. Part of the LDH activity was then soluble in 2.6M ammonium sulfate. After 36 to 48 hours’ incubation at 4° to 10° C. little or no activity was recoverable when the ammonium sulfate concentration was made 2.6M, although the solution still had as much or more LDH activity than was taken originally when assayed with diethyldithiocarbamate in the assay mixture.

LDH preparations from bovine lenses which have been subjected to column chromatography and then treated with HgCl₂ solution for 36 to 48 hours have the same protein distribution after disc electrophoresis on acrylamide gels as was found for the LDH before addition of HgCl₂ solutions or 6 hours after addition of HgCl₂. Duplicate electropherograms obtained immediately after mixing and 6 hours after mixing, when stained for LDH activity with addition of diethyldithiocarbamate to the staining solution, revealed LDH activity at the same location at which protein was found. However, the duplicate electropherograms which were run 36 to 48 hours after addition of HgCl₂, similarly stained for LDH activity, showed no line of LDH activity where the protein was found. A diffuse stain with a sharp boundary on the positive electrode side, but fading off toward the origin, was sometimes found. All efforts to isolate the lens LDH activity which is soluble in 2.6M ammonium sulfate solutions have failed so far.

REFERENCES