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Increased Specific Activity and Formation of an Inhibitor from the LDH_5 Isozyme of Lactate Dehydrogenase*

Albert H. Gelderman and Andrew C. Peacock

ABSTRACT: The specific activity of the LDH_5 isozyme of crystalline rabbit muscle lactate dehydrogenase can be increased 200% by means of sucrose density gradient centrifugation or Sephadex G-200 molecular sieving of the enzyme in the presence of low concentrations of β-mercaptoethanol. Also separated by the procedure is a denser protein fraction that is inhibitory to LDH_5. In contrast to the original LDH_5, the LDH_5 that is activated by β-mercaptoethanol treatment contains no detectable reduced nicotinamide–adenine dinucleotide X (NADH-X), while the inhibitor fraction contains approximately three times as much presumed NADH-X per mole of protein. The addition of excess NADH to the sucrose density gradient prevents the separation of LDH_5 into the activated and inhibitory fractions. The LDH_1 isozyme is not affected by the β-mercaptoethanol treatment and is not inhibited by the LDH_5 inhibitor fraction.

We have found that in the presence of low concentrations of β-mercaptoethanol there are substantial and significant changes in the properties of the molecule of LDH_5 isozyme of lactate dehydrogenase. In the studies described here it is shown that LDH_5 in the presence of β-mercaptoethanol can be separated by sucrose density gradient sedimentation and Sephadex G-200 chromatography into essentially two protein fractions, one with a high specific activity and the other with no enzymatic activity and an inhibitory action to LDH_5.

Methods

Rabbit muscle lactate dehydrogenase, substantially free of pyruvate kinase, was obtained as a three-times-crystallized preparation from Sigma Chemical Co. The enzyme was dialyzed against 0.001 M Tris-HCl buffer, *p*H 7.5, and separated into the five isozyme fractions by DEAE-cellulose chromatography as previously described (Gelderman *et al.*, 1965). LDH_5 is defined as the cathodal isozyme by electrophoresis or that isozyme not adsorbed to DEAE-cellulose. A value of 1.45/mg

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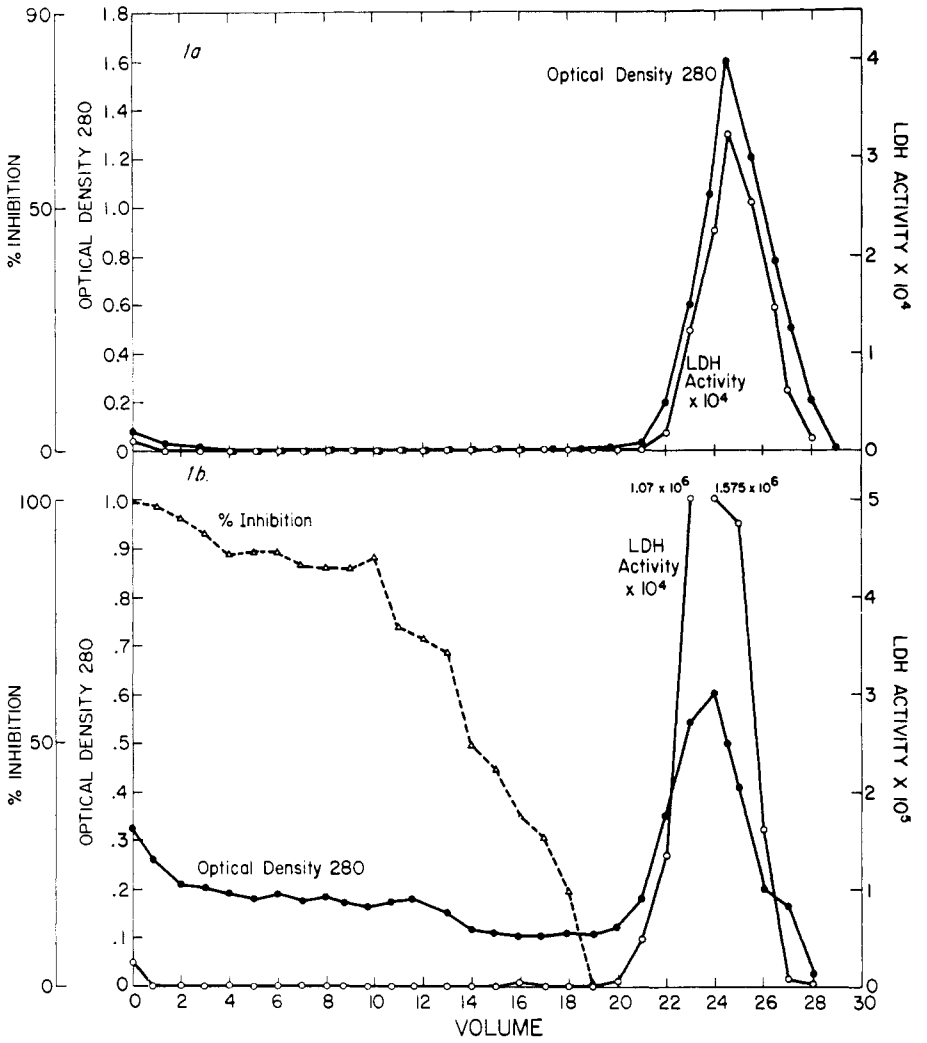

FIGURE 1: Sucrose density gradient (20–4%) centrifugation of LDH_5 with and without the addition of β-mercaptoethanol. (a) Sucrose density gradient centrifugation of LDH_5 without β-mercaptoethanol. The centrifuge tube contained a linear sucrose gradient from 20 to 4% in 0.01 M Tris-HCl buffer, *p*H 7.5. The 0.5-ml sample applied contained 2.0×10^6 units of LDH and $6.090 A^{280}$ units (8.53 mg LDH) of protein. The protein recovery was 100% and the LDH recovery was 9.2×10^5 units of LDH activity (46%). Tube 0 always represents the "redissolved" pellet fraction. (b) Sucrose density gradient centrifugation of LDH_5 with β-mercaptoethanol added to the buffer so that the concentration was 0.005 M. The sucrose gradient was identical with part (a), except for incorporation of β-mercaptoethanol, 0.005 M. The sample was the same as (a). The protein recovery was 100% and the LDH activity recovered was 4.3×10^6 units or a 213% recovery. The data is corrected for β-mercaptoethanol absorption.

per ml was used as the specific absorbance of the crystalline enzyme (Hakala *et al.*, 1956). The molecular weight of the enzyme was taken as 135,000 (Neilands, 1952). Enzyme activity was determined by the method of Henry *et al.* (1960). A unit of activity was defined as that activity which would cause a decrease of the absorbance of NADH[1] at 340 mμ of 0.010 per minute. The specific activity of the LDH_5 isozyme was nearly identical with that crystallized by Nisselbaum and Bodansky (1963). Solutions containing β-mercaptoethanol were either dialyzed or greatly diluted prior to assay.

The inhibitory activity of a fraction was assayed by measuring the per cent loss of activity of a known amount of LDH after incubation with the fraction to be tested for inhibitor activity. Unless stated otherwise, 100 units of β-mercaptoethanol-treated LDH_5 were incubated in Tris-HCl buffer at *p*H 7.5 for 16 hours at 4° with the fraction to be tested for inhibitor activity. Because the A^{280}, and hence the protein concentration of the various fractions, was different, the volume of inhibitor solution was adjusted so that the product (volume $\times A^{280}$) was equal to 3.0×10^{-3} in each case. The same amounts of

[1] Abbreviations used in this work: NADH, reduced nicotinamide-adenine dinucleotide; LDH, lactate dehydrogenase.

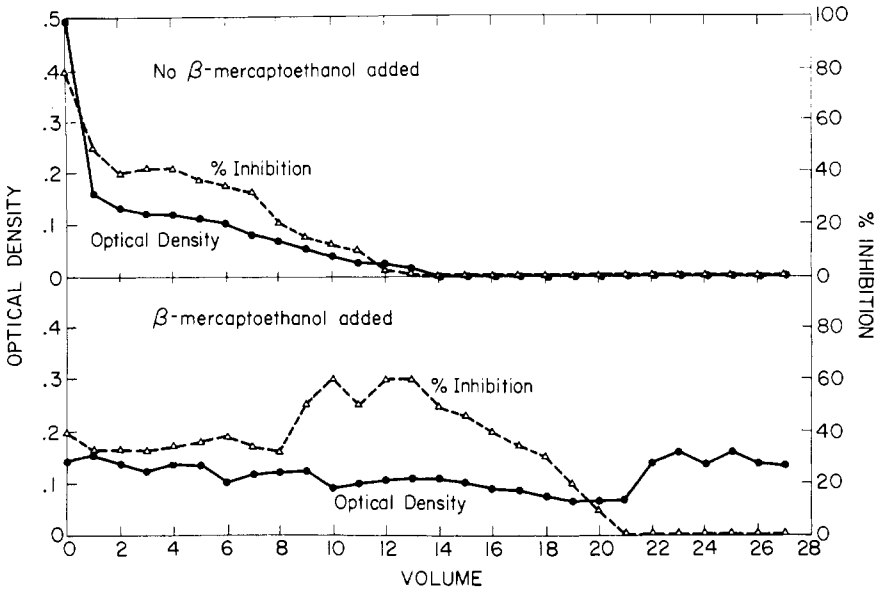

FIGURE 2: Sucrose density gradient (20–4%) centrifugation of the "inhibitor" fraction (tubes 1–7, Figure 1b, pooled, dialyzed, and lyophilized). (a, top) Sucrose density gradient centrifugation of inhibitory fraction without β-mercaptoethanol (same conditions as Figure 1a). Recovery of both inhibitory activity and protein was poor owing to insoluble aggregates. (b, bottom) Sucrose density gradient centrifugation of same material in the presence of β-mercaptoethanol. (The conditions are the same as those in Figure 1b.)

β-mercaptoethanol-treated LDH_5 and the fraction to be tested were incubated in separate tubes under the same conditions. After incubation, the three tubes were assayed for LDH activity as described above. If the fraction to be assayed for inhibitor activity had LDH activity, this was subtracted from the activity of the combined fractions in order to determine the per cent inhibition.

Sucrose Density Gradient. All operations were performed at 4°. The gradient was prepared with an apparatus similar to that described by Britten and Roberts (1960). Sucrose (14 ml of 4% w/v) in 0.01 M Tris-HCl buffer, *p*H 7.5, was mixed with 14.5 ml of a 20% sucrose solution in the same buffer so as to form a linear gradient. In those cases where β-mercaptoethanol was added to the sucrose solutions, the concentration was 0.005 M. The sample for analysis was 0.5 ml. The tubes were centrifuged in a Spinco Model L ultracentrifuge at 23,500 rpm for 16.5 hours in a SW 25 (Spinco) rotor. At the end of the run the tubes were pierced at the bottom with a 16-gauge needle and 1-ml fractions were collected. The sucrose concentrations determining the gradient were determined by measurement of the refractive index. The tubes were assayed for volume, protein, and LDH activity.

Fractionation on Sephadex G-200. The column of Sephadex G-200 (Pharmacia Co.), 200–400 mesh, was prepared essentially as described by Flodin and Killander (1962). The column measured 3 × 60 cm and was eluted by means of a constant head of pressure of 7 cm above the base. The flow rate was 30 ml/hour at 25°. The 2.0-ml sample volume was layered on the surface of the column and was washed in and eluted with 0.1 M Tris-HCl buffer, *p*H 7.2. The effluent was collected in 3-ml portions using an automatic fraction collector.

Polyacrylamide-gel electrophoresis was done by the method of Davis (1964). Starch-gel electrophoresis was performed as previously described (Gelderman, 1964).

Perchloric acid (0.5 M) precipitation and spectral analysis of the supernatant was done according to Wieland *et al.* (1962). Absorption spectra were determined on a Cary Model 15 spectrophotometer.

Results

Sucrose Density Gradient Centrifugation. The results of the sucrose density gradient centrifugation of crystalline rabbit muscle LDH_5 are shown in Figure 1. The LDH, when not in the presence of β-mercaptoethanol (Figure 1a), was found in a single sharp peak of protein and LDH activity. The protein recovery was 100%, but the LDH activity recovery was 46%. When the same sample of LDH_5 was centrifuged in the presence of β-mercaptoethanol (Figure 1b), the LDH was separated into two fractions with different properties. The major fraction ("enhanced" fraction), found in a peak at the same sucrose density as the LDH in Figure 1a, had LDH activity with a specific activity 213% higher than that of the LDH applied. The remainder of the protein in the tube was distributed in the denser portions of the tube and was inhibitory to the LDH from the active peak. The total recovery of the protein was 100%, and the total recovery of the LDH

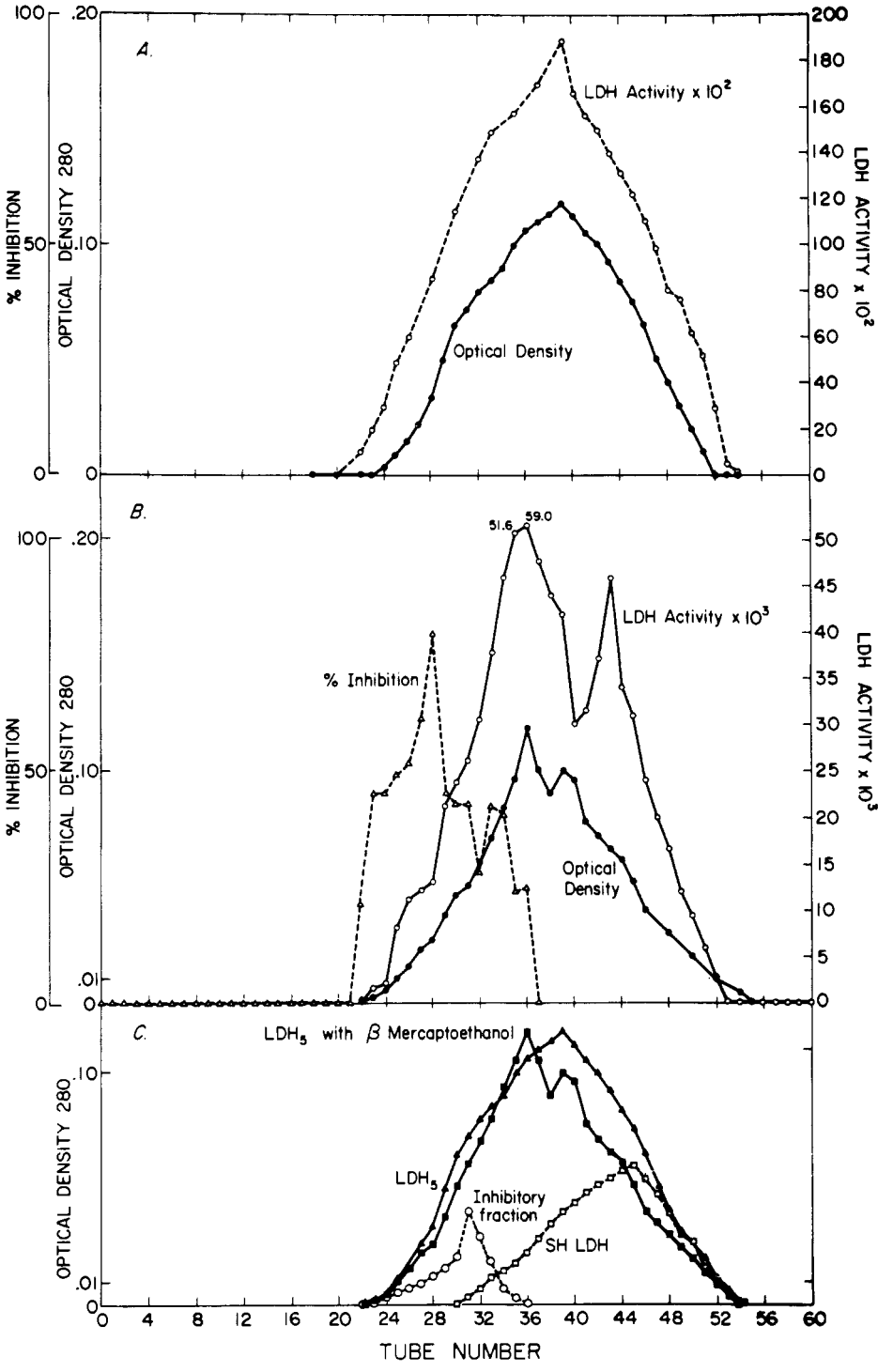

FIGURE 3: Sephadex G-200 chromatography of LDH_5. (A) Sephadex G-200 chromatography of LDH_5 without β-mercaptoethanol. LDH activity (4.7×10^5 units) and $1.4A^{280}$ units of protein were applied in a 2.0-ml sample. There were $1.4A^{280}$ units of protein and 2.6×10^5 units of LDH_5 recovered. (B) Sephadex G-200 chromatography of LDH_5 with 0.005 M β-mercaptoethanol added to the eluting buffer. The recovery of LDH activity was 163% or 7.9×10^6 units. The inhibitory activity of the fractions is plotted as per cent inhibition. (C) Molecular-sieve resolution of the inhibitory fraction (-O-O-O-O-), the "enhanced" LDH_5 (—SH LDH) fraction (□-□-□), LDH_5 (▲-▲-▲), and LDH_5 in the presence of 0.005 M β-mercaptoethanol (■-■-■). Lines refer to continuous recording of the absorbance at 280 mμ taken on a Gilford recording spectrometer.

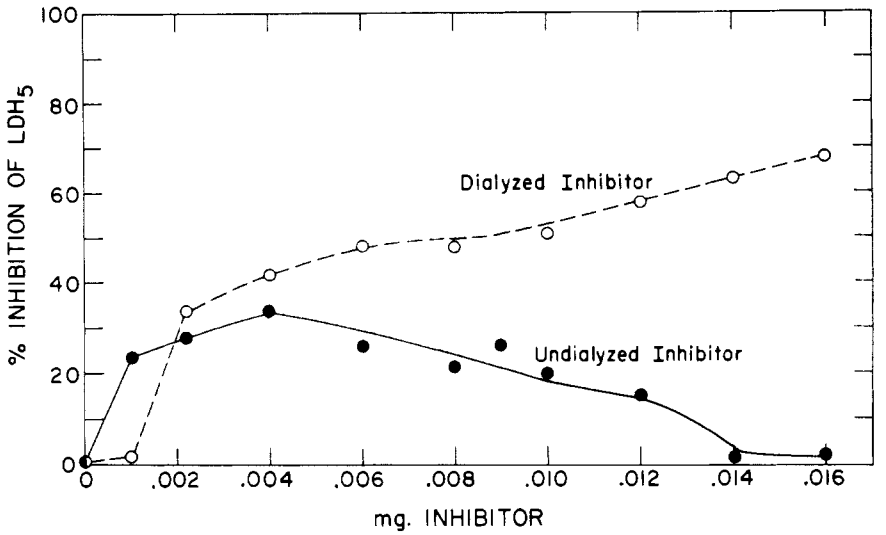

FIGURE 4: The per cent inhibition of LDH_5 in response to increasing quantities of dialyzed and undialyzed inhibitor. The undialyzed inhibitor is in a solution of approximately 10% sucrose in 0.01 M Tris-HCl buffer, *p*H 7.5, and 0.005 M β-mercaptoethanol. The dialyzed inhibitor was dialyzed for 56 hours against 0.01 M Tris-HCl, *p*H 7.5. The inhibitor and enzyme were incubated for 12 hours at 4°.

activity was 213%. When LDH_5 was centrifuged in a sucrose gradient with β-mercaptoethanol and, instead of collecting the fractions, the gradient was mixed, the recovery of LDH activity was 100%.

The tubes with inhibitory activity (tubes 1–17, Figure 1b) were pooled, concentrated by lyophilization, and reapplied to the sucrose density gradient, with and without β-mercaptoethanol. In the absence of β-mercaptoethanol some of the inhibitory fraction formed a flocculant precipitate that settled to the bottom of the tube (Figure 2a). In the presence of β-mercaptoethanol (Figure 2b), the same material was redistributed over the entire density-gradient tube, and a small diffuse peak of inactive protein was found at the sucrose density where the peak of LDH had been found. Inhibitor activity was found in the denser fractions.

When the LDH of enhanced specific activity from Figure 1b (tubes 21–27) was rerun on the sucrose gradient with and without β-mercaptoethanol, there was no change in the LDH specific activity and no inhibitor fraction was found.

In similar experiments the density-gradient centrifugations with and without β-mercaptoethanol were performed in the presence of added NADH (6 moles NADH/mole LDH_5). In the experiments in which there was an excess of NADH, density-gradient centrifugation failed to show an increase in specific activity or the appearance of an inhibitor.

LDH_1 was centrifuged with and without β-mercaptoethanol; there was a sharp peak of protein associated with LDH activity at the same sucrose density as that of the LDH_5. In contrast to the experiments in which LDH_5 was used, the presence of β-mercaptoethanol did not result in an increase in specific activity of the LDH_1 nor did separation of the protein into an inhibitory fraction and an active fraction occur.

Chromatography of LDH_5 on Sephadex G-200. After "molecular-sieve" fractionation of LDH_5 on Sephadex G-200 with no β-mercaptoethanol added, the LDH was found in a single peak with 55.4% of the LDH activity recovered (Figure 3a). When the eluting buffer contained 0.005 M β-mercaptoethanol, two peaks of protein and LDH activity were recovered (Figure 3b). The peak of LDH activity that was most retarded by the column (higher tube numbers) had twice the specific activity of the peak passing through the column more rapidly. Further, the less-retarded peak was associated with inhibitor activity. The total LDH recovery was 167%, and the protein recovery was 100%.

The inhibitor fraction and the fraction of LDH with an enhanced specific activity (SH-LDH) prepared by sucrose density gradient centrifugation (Figure 1b) were each chromatographed on the Sephadex G-200 column (Figure 3c). The "inhibitor" fractions passed through the column more rapidly than the "enhanced" LDH fraction.

Properties of the Inhibitor. The "inhibitor" was able to withstand continuous dialysis against 0.005 M Tris-HCl buffer for 48 hours without loss of inhibitory activity. When increasing amounts of undialyzed inhibitor were added to 100 units of LDH, there was initially an increasing amount of LDH inhibition, but further additions of inhibitor to the enzyme resulted in a progressive decrease of inhibition (Figure 4). When the same experiment was repeated with dialyzed inhibitor the inhibition was slightly greater and increased with increasing amounts of inhibitor added (Figure 4).

The time required for inhibition to develop with the undialyzed and dialyzed inhibitor differed. When the

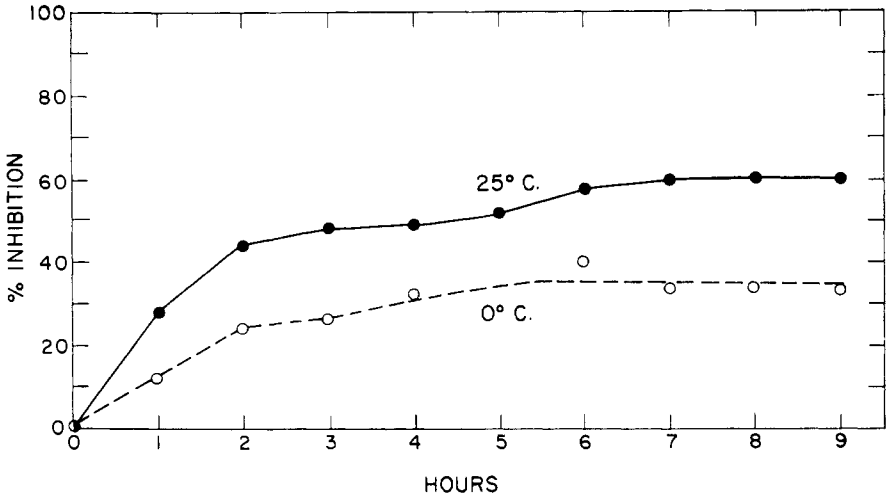

FIGURE 5: The rate of inhibition of LDH_5 by dialyzed inhibitor at 0 and 25°. Dialyzed inhibitor (0.010 mg) was incubated with 0.002 mg of β-mercaptoethanol-treated LDH at 0 and 25°.

per cent inhibition using undialyzed inhibitor was studied as a function of time, there was no detectable inhibition observed in the first 9 hours, a maximum inhibition in 12 hours, followed by a loss of inhibition in 24 hours. The dialyzed inhibitor reacted faster (Figure 5) and reached a stable maximum in 9 hours. The rate and degree of inhibition was much greater at 25° than at 0°.

The "inhibitor" is relatively heat stable; only 50% of the activity was lost after heating at 100° for 15 minutes. The "inhibitor" had a 30% greater inactivation effect on the "enhanced" LDH_5 (that treated with β-mercaptoethanol) than on the untreated LDH_5. There was no inhibition of the LDH_1. Reduced glutathione did not reverse the inhibition of the LDH_5. The "inhibitor" fraction had the same electrophoretic mobility as the LDH_5 on polyacrylamide-gel electrophoresis.

Properties of the "Enhanced" LDH Fraction. The LDH fraction isolated by sucrose-gradient centrifugation in the presence of β-mercaptoethanol was identical to untreated LDH_5 with respect to electrophoretic mobility on polyacrylamide and starch gel. It was also identical to untreated LDH_5 in respect to the Michaelis constants for pyruvate and NADH, and in susceptibility to heat inactivation. The presence of β-mercaptoethanol in concentrations less than 0.5 M had no effect upon the enzymatic activity.

The enzymes were noted to differ in respect to cold inactivation. When the "enhanced" LDH was frozen in 0.1 M Tris-HCl buffer, *p*H 7.9, at −20° for 96 hours, there was no loss in activity, while untreated LDH_5 lost 50% of its activity.

Spectral Analysis. After recentrifugation of the inhibitor in the presence of β-mercaptoethanol (Figure 2b), the fractions were pooled into three equal parts (tubes 1–8, 9–20, 21–27), and the spectra of these fractions were determined. The 265/310 mμ ratio of the three inhibitor fractions, the untreated LDH_5, and the β-mercaptoethanol-treated LDH_5 (Figure 1b) were determined. Ratios of the untreated LDH_5 and the pooled inhibitor fractions, 21–27, were similar (3.22 and 3.15, respectively). The "inhibitor" pooled from tubes 9–20 and that pooled from tubes 1–8 had ratios of 4.09 and 4.69, respectively. The "enhanced" LDH_5 had the lowest 265/310 mμ ratio (2.21).

The spectra of the supernatant fractions of the inhibitor and LDH_5 after 0.5 M perchloric acid precipitation are shown in Figure 6. These spectra resemble that of the secondary acid product of NADH-X (Wieland *et al.*, 1962). In addition, other properties of the inhibitory material, i.e., not removed from the enzyme by ammonium sulfate precipitation, dialysis, or ion-exchange chromatography, are consistent with those reported for NADH-X, and suggest that the inhibitor contains NADH-X. Using the extinction coefficient reported for NADH-X, it was calculated that there was 1 mole of NADH-X to 1 mole of untreated LDH_5. On the same basis, there were 3 moles of NADH-X associated with 1 mole of inhibitor (if the molecular weight of the inhibitor and its extinction coefficient are assumed to be the same as that of LDH [135,000]). In contrast, there was no evidence for NADH-X in the enhanced LDH_5 fraction.

Discussion

We have found that preparations of LDH_5, largely physically homogeneous by electrophoresis, ion-exchange chromatography, and sucrose density centrifugation, exhibit substantially higher specific activity after density-gradient centrifugation in the presence of low concentrations of β-mercaptoethanol. The product resulting from this treatment differs from the original LDH_5 by an enhanced specific activity, a lower content of NADH-X, a resistance to cold inactivation, and a

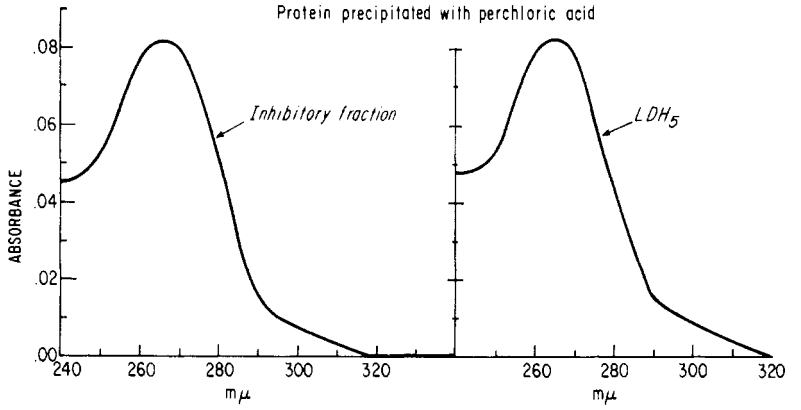

FIGURE 6: Spectra of nonprotein fraction of LDH_5 and inhibitory fraction. LDH_5 (4.35 mg) and 1.6 mg of inhibitor were precipitated with perchloric acid, 0.5 M final concentration.

greater retardation on Sephadex G-200. The activation of LDH_5 is accompanied by the appearance of a second fraction, the inhibitory fraction, which is characterized by its lack of LDH activity, apparently higher molecular weight, a greater tendency to aggregate, and an inhibitory effect on both LDH_5 and the enhanced LDH fraction.

These phenomena may be the result of releasing inhibitory material previously attached to the tetramer structure. If this material were attached to the molecule through the mediation of —SH bonds, the forces holding the inhibitor to the LDH_5 molecule might be sufficiently weakened by the competitive action of β-mercaptoethanol to permit the inhibitor to be removed by ultracentrifugation. Alternatively, the inhibitory material may be more integrally bound to the LDH_5 molecule, possibly in the form of a "defective" monomer. In this case, a more extensive rearrangement of the LDH_5 molecule would be indicated, in which the combined action of β-mercaptoethanol and ultracentrifugal separation might favor the partial dissociation of the tetramer structure, followed by centrifugal removal of the inhibitory material and recombination of monomers into more active tetramers.

The enhanced LDH_5 has apparently the same density as the original material (Figure 1), but may be somewhat smaller (Figure 2c). The material separated from the LDH_5 during the rearrangement is grouped into polydisperse molecules somewhat larger (Figure 3) and more dense (Figure 2) than the parent material. The physical properties of the inhibitor units are strongly dependent upon the presence of β-mercaptoethanol (Figure 2). In the presence of β-mercaptoethanol they form a heterogeneous mixture with respect to density, but in the absence of β-mercaptoethanol the units combine and sediment to the bottom of the density gradient.

The changes in the LDH_5 molecule produced by β-mercaptoethanol are partially reversible. The heavier, more dense inhibitor fraction can recombine with the enhanced LDH_5 with a consequent loss of LDH activity. The recombination proceeds better in the absence of β-mercaptoethanol.

Thus, in the absence of β-mercaptoethanol, there is a stable structural complex (which persists after many recrystallizations, dialysis, and ion-exchange chromatography) involving the integration of the "inhibitor" and the "enhanced" LDH_5. The presence of β-mercaptoethanol apparently favors the formation of two species: an LDH_5 of increased specific activity, and a separate inhibitory fraction which then polymerizes.

NADH-X previously has been found in many lactate dehydrogenases in a ratio of 1 mole NADH-X to 1 mole LDH protein, and, in excess, has been shown to inhibit LDH activity (Wieland *et al.*, 1962). The amounts of presumed NADH-X found in the untreated LDH_5, the enhanced LDH_5, and the inhibitory fraction are quite different. The untreated LDH_5 contains 1 mole of NADH-X/mole protein. The inhibitory fraction contains perhaps as much as 3 moles of NADH-X/mole protein (assuming an extinction coefficient similar to that of LDH). The enhanced LDH_5 appears to contain no NADH-X.

Since NADH-X is known to have inhibitory properties to LDH, the substantially greater content of NADH-X in the inhibitory fraction is consistent with its inhibitory properties. Further, the absence of NADH-X in the enhanced LDH_5 fraction may account, at least in part, for the greater specific activity observed.

Our results agree with those of Zondag (1963), that the LDH_5 frozen for 96 hours at −20° is partially inactivated. In contrast, the enhanced LDH_5 freed of units containing NADH-X is stable under these conditions. Since freezing of the isozymes was reported by Markert (1963) to result in dissociation into monomeric units, repolymerization and reorganization of the LDH_5 in the presence of units containing NADH-X might account for the loss of activity. The cold stability of the "enhanced" LDH_5 is possibly due to an absence of "inhibitor" units containing NADH-X.

The β-mercaptoethanol-mediated changes in the

LDH_5 molecule were prevented by the addition of an excess of NADH to the density gradient. This would explain why Bonavita and Guarneri (1963) found no effect of β-mercaptoethanol on the kinetic behavior of LDH since NADH was used in a substantial excess.

The role of an excess of NADH in preventing the β-mercaptoethanol-dependent changes in LDH_5 may be due to a tighter binding of the LDH_5 monomers. This would agree with the data of Fritz and Jacobson (1963), who observed fewer bands of LDH_5 activity when NADH was added in excess during electrophoresis performed in the presence of β-mercaptoethanol, and agrees with Zondag's (1963) finding that NADH protects against cold inactivation. It is interesting that the structure of LDH_1 is sufficiently different from the LDH_5 so that β-mercaptoethanol produced no detectable changes.

The physiological significance of these properties of LDH_5 cannot be assessed here. However, should similar reactions occur *in vivo*, they would have an important significance in the regulation of LDH activity.

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The Role of Metal Ions in the Pyruvic Kinase Reaction*

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ABSTRACT: The velocity of the pyruvate kinase reaction has been measured over a wide range of concentrations of adenosine diphosphate (ADP), Mg^{2+}, and K^+. The data have been analyzed in terms of the equilibrium concentrations of the simple and the complex ions present in the solution. It is concluded that $MgADP^-$ is the specific substrate required, while K^+ functions to activate the enzyme. The data fit the mechanism proposed by Reynard and co-workers (A. M. Reynard, L. F. Haas, D. O. Jacobsen, and P. D. Boyer [1961], *J. Biol. Chem. 236*, 2277), except that the restriction of "equilibrium kinetics" does not appear to be justified.

The pyruvic kinase of muscle has been extensively studied (Boyer, 1962). In addition to requiring magnesium ion for activity (Lohman and Meyerhof, 1934), it represents one of the rare instances of a simple system which exhibits an absolute requirement for a monovalent cation (Boyer *et al.*, 1942; Boyer *et al.*, 1943; Lardy and Zeigler, 1945). Karchmar and Boyer (1953) have shown that rubidium or ammonium ions, but not sodium, can replace the activation by potassium. Reynard *et al.* (1961) have presented evidence that the enzyme possesses a binding site for each of its two substrates, and that the phosphoryl group is transferred directly from the donor to the acceptor. Evidence against an intermediate phosphoryl enzyme has been presented by Haas *et al.* (1961). From magnetic resonance studies of the Mn^{2+} activated system, Cohn (1963)

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