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A Radiotracer Probe to Study Metal Interaction with Human Lactate Dehyrogenase Isoenzymes[1]

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The electrophilic Ag^+ ion was found to destroy completely the enzymatic activity of lactate dehydrogenase isoenzyme LDH-1 while other transition metal ions reduced its activity in varying degrees. A radiotracer probe involving ^{110m}Ag-labeled silver ion was used to understand the mechanism of denaturation of LDH and also to determine the number of active sites, if any, for substrate binding with the enzyme. Purified LDH-1 was reacted with ^{110m}Ag-labeled silver ion and the mixture was passed through the sephadex G-75-120 gel to separate the ^{110m}Ag-LDH complex that might be formed during the reaction. The resulting elution curve revealed that a stable complex was formed. From the total radioactivity of ^{110m}Ag bound LDH, the specific activity of labeled Ag^+ and the amount of LDH used the ratio of the number of moles of Ag^+ reacted with 1 mol of LDH was computed. This was found to be approximately 4.0, indicating that there are four binding sites in LDH, probably one on each subunit. Kinetic studies of LDH catalysis of L-P reaction in the presence and absence of Ag^+ ion suggest that silver ion is involved in competitive inhibition and that the interaction conforms to the "lock-and-key" model. The inhibition of catalysis by other metals is presumably of a noncompetitive type.

KEY WORDS: Lactate dehydrogenase enzyme; radioactive probe; active sites; inhibition of catalysis.

1. INTRODUCTION

Many metals interact with enzymes and play an important role in controlling enzymatic activity. Vallee (1980, 1981) has discussed the role of metals as probes in the study of enzyme conformation and function. Some enzymes are characterized as metalloenzymes since the metal is an integral part of these enzymes and participates in the actual catalytic process (Vallee, 1980). Others are inhibited or enhanced in their activities by interaction with metals, especially divalent transition metals (Vallee, 1980; Foreman and Niehaus, 1985). Denaturation of enzymes also occurs in the presence of certain organic or biochemicals (Sabato and Kaplan, 1964; McLoughlin and Howell, 1987). Many theories and models have been proposed in

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the past to describe the mechanism of catalysis by metallo- and nonmetallo-enzymes and its inhibition by various inhibitors—the most important of which is the "lock-and-key model," which is accepted by many scientists (Holmquist and Vallee, 1979; Jenchs, 1975). In the past, spectroscopic properties of the interacting metal ions, EPR studies, viscosity and atomic absorption measurements, labeled affinity probes, etc., were employed to study the mechanism of inhibition or enhancement of enzymatic activities, and also to confirm their active sites (Vallee, 1980; Culp *et al.*, 1985; Senior *et al.*, 1980; Ikebe and Hartshorne, 1985; Daggett *et al.*, 1985).

During our previous investigation on the development of radioimmunoassays for lactate dehydrogenase (LDH) isoenzymes (Menon *et al.*, 1985) it was once observed that the presence of Ag^+, an electrophilic transition metal ion, in the LDH sample completely destroys its activity. This fact led us to investigate the possibility of using ^{110m}Ag-labeled Ag^+ ion as a radiotracer probe to determine the number of active sites in LDH enzyme, and also to study the mechanism of inhibition of its activity by silver. In this paper we report our findings on the use of a simple radiotracer technique for the determination of the number of active sites in LDH enzyme, and also try to explain the mechanism of inhibition of its activity by Ag^+ and probably by some other transition metal ions.

2. EXPERIMENTAL PROCEDURES

2.1. Materials and Equipment

Human heart muscles separated from autopsy material (Memorial Medical Center, Savannah, Georgia) were used to isolate LDH-1 (H_4) isoenzyme. Unswollen DEAE-cellulose anion-exchange resin (Sigma) equilibrated with tris (hydroxymethyl) aminomethane hydrochloride (TRIS-HCl) was employed to isolate and purify the LDH isoenzymes from myocardial extract by anion-exchange chromatogrphy. The protein assay kit supplied by Bio-Rad laboratories was used to assay LDH in the purified sample. Radiotracer ^{110m}Ag (255 d half-life) mixed with inactive silver (0.61 mg/ml) in the form of $AgNO_3$ was purchased from Amersham Corporation. Sephadex G-75-120 gel column (15 cm × 0.7 cm) pre-equilibrated with phosphate-lactate buffer (0.01 *M* phosphate, 0.15 *M* lactate, *p*H 7.6) was used for size exclusion chromatography. All other chemicals used in this study were of reagent grade purity. The equipment for the study include 3″ × 3″ NaI(Tl) well crystal gamma ray spectrometer and accessories furnished by Canbera, Perkin Elmer model Lambda 3 UV visible spectrophotometer, Helena's Titan Gel electrophoresis apparatus and Cliniscan II densitometer, and Gilson's microfraction collector.

2.2. Isolation and Purification of LDH-1 Isoenzyme

LDH isoenzymes were isolated from myocaridal extract by anion exchange chromatography using a *p*H-coupled salt gradient elution method described by Menon *et al.* (1985). The LDH-1 fraction was pooled and repurified by the same chromatographic technique until its purity was confirmed by electrophoresis.

2.3. Measurement of the Concentration and Activity of LDH-1

The concentration of protein in the purified LDH-1 enzyme fraction was measured using Bio-Rad protein determination kit and the procedure recommended by Bradford (1976). The assay is based on the observation that λ_{max} for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465–595 nm when it binds with the protein. Enzymatic activity was determined at 37 ± 0.5°C using the method of Walker *et al.* (1956) involving LDH-catalyzed conversion of lactate to pyruvate (L-P) in the presence of NAD^+ (Menon *et al.*, 1983). Final concentrations in mmol L^{-1} of each reagent in the assay mixture (2.8 ml of substrate with $NAD^+ + 0.2$ ml of sample) were the following: sodium phosphate buffer (pH 8.8) 50.0, sodium lactate 50.0, NAD^+ 5.0.

2.4. Studies on the Interaction of Selected Metals with LDH-1

The enzymatic activity of LDH-1 was measured in the absence and presence of various metal ions. A 2.8 ml of the substrate containing the coenzyme, NAD^+ was vortexed with 0.2 ml of the diluted enzyme. The final concentrations of the reagents in the mixture were the same as before. The absorbance of the mixture at 340 nm (λ_{max} for NADH) was measured at 20 sec after mixing, and also every 30 sec thereafter for 2 min. The activity was calculated using the expression:

$$A(\text{U/ml}) = (\Delta A/\text{min})/0.001 \times V \tag{1}$$

where ΔA is the initial change in absorbance per minute and V is the volume of the sample used for assay (Menon *et al.*, 1983). Fifty microliters of each metal ion of appropriate concentration was treated with the same volume of the substrate and LDH-1, then vortexed and assayed for the enzymatic activity.

Percent reduction in he activity computed from the measurements gave the extent of inhibition of LDH catalysis of L-P reaction by the metal interaction.

2.5. Determination of the Active Sites of LDH-1 with ^{110m}Ag tracer

An aliquot of the stock solution of ^{110m}Ag (~1 mCi) was diluted with deionized water and 25 μl of the diluted tracer was counted in the gamma ray spectrometer. With the energy window gated at 200, and with full window opening on the single channel analyzer, the characteristic gamma radation (0.658 Mev, 98%) was counted. From the known amount of inactive Ag^+ in the original tracer and the counting rate of the standard, the specific activity (cpm/mmol) was computed. One milliliter of the stock solution of purified LDH-1 (4.57×10^{-6} M) or a fraction of it was vortexed with excess of radiolabeled Ag^+ ion and passed through the sephadex G-75-120 gel that was pre-equilibrated with the phosphate-lactate buffer (PLB). After the complete absorption of the mixture, it was eluted with PLB and 0.6 ml fractions were collected in tubes using Gilson's automatic fraction collector. An aliquot of 0.4 ml from each fraction was transfered to a polyethylene counting tube and its radioactivity was counted in the gamma ray spectrometer. A plot of the relative net counting rate, after the substraction of the background, with the fraction number resulted in an elution curve with two peaks: one due to the silver ion-bound

LDH-1 and the other due to the unbound Ag^+ ion. This elution curve is depicted in Fig. 1a. The total net activity of all fractions or peak area under the Ag^+-LDH-1 peak was computed by summing their net activities. The number of mmoles of Ag^+ bound to the number of mmoles of LDH-1 originally present in the reaction mixture was then computed from the specific activity of ^{110m}Ag tracer used for the reaction and the total net activity of Ag^+ bound LDH-1. From these values the ratio of the silver ions bound per molecule of LDH-1 or the number of active sites where Ag^+ ion is presumably bound was determined.

The combined eluate from fractions under the first peak was passed through the sephadex gel column, and elution was carried out with the same phosphate-lactate buffer. The resulting elution curve is depicted in Fig. 1b. In another experiment the

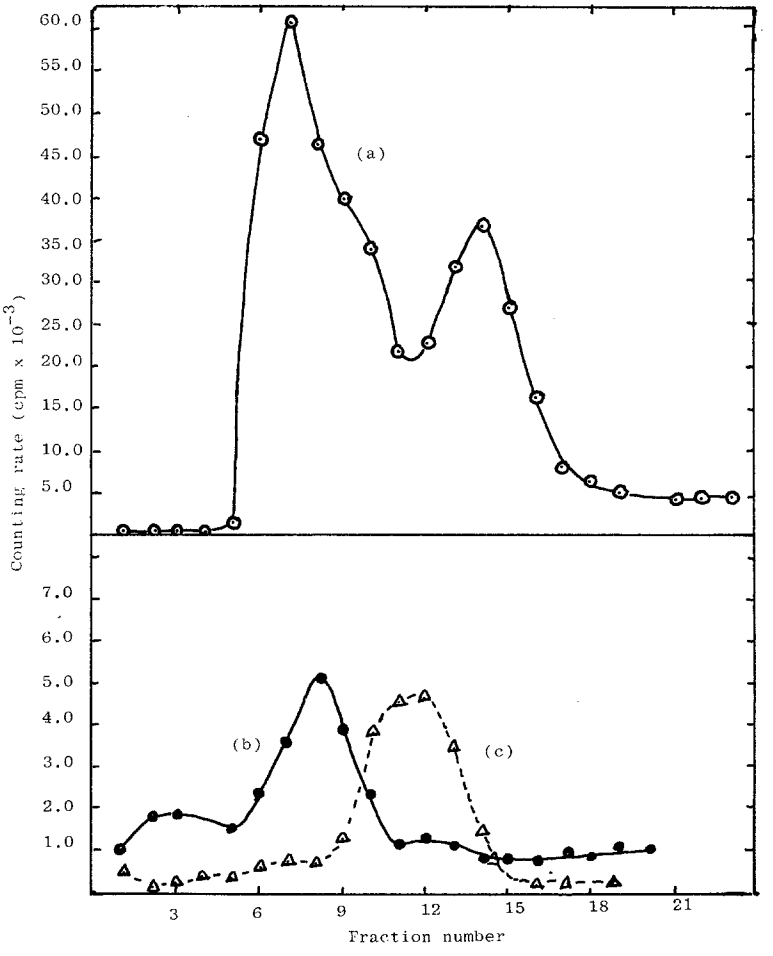

Fig. 1. Elution pattern of (a) ^{110m}Ag bound LDH-1 with excess of $^{110m}Ag^+$ tracer, (b) pure ^{110m}Ag bound LDH-1, and (c) Ag-LDH-1 complex reacted with EDTA from size-exclusion chromatography.

combined eluate containing the enzyme-metal complex (2.4 ml) was mixed with 1 ml of 0.05 *M* EDTA (*p*H 7.6) and passed through the sephadex column. The mixture was eluted with the same PLB and fractions were collected as before. The elution curve obtained from this experiment is shown in Fig. 1c. In another experiment the eluted fractions that contained the enzyme metal complex were examined for the enzymatic activity of LDH. These fractions recorded very little or no activity at all, indicating that the substrate cannot compete with the bound Ag^+ for interaction with LDH.

2.6. Kinetics of LDH Inhibition by Silver Ion

To determine whether silver ion is involved in competitive, noncompetitive, or uncompetitive inhibition, rate measurements were made in the presence and absence of silver ion. The initial velocities of L-P reaction at constant concentration of LDH-1 and NAD^+ but with different concentration of the substrate (lactate) were first determined in the absence of silver ion. Similar measurements were made in the presence of 6.62×10^{-8} *M* Ag^+ ion in the mixture. Lineweaver-Burk plots relating the reciprocal velocity to reciprocal substrate concentration will permit the distinction among the three types of competition.

Using the same concentration of the substrate, NAD^+, and the enzyme, the activity measurement was made as a function of increasing concentration of the inhibitor, Ag^+ ion. The activity was found to decrease linearly with the increase in the concentration of the inhibitor within the range chosen for the experiment.

3. RESULTS

The results of the metal-inhibition studies on the LDH-1 catalysis of L-P reaction are presented in Table I. It can be seen from this table that Ag^+ ion completely destroys the LDH activity even at a lower concentration than the rest. Vanadium and mercury also inhibit the catalysis significantly but at higher concentrations. It appears that these metals, excluding Ag^+, take part in uncompetitive inhibition that involves their interaction with the enzyme-substrate complex. Figure 1 depicts the elution curves of: (a) reaction mixture containing LDH-1 and ^{110m}Ag-labeled Ag^+ ion; (b) the combined fraction containing the metal enzyme complex; and (c) the metal-enzyme complex and EDTA mixture. Since in size-exclusion chromatography the macromolecules are eluted first followed by the smaller molecules, it is obvious from this figure that the first peak resulted from the elution of ^{110m}Ag-labeled Ag-LDH-1 complex (Fig. 1a and the second from the elution of unreacted ^{110m}Ag. This fact was confirmed by the results shown in Fig. 1b wherein the elution pattern of combined fraction corresponds to the silver-enzyme complex. However, Fig. 1c demonstrates that Ag^+-EDTA complex is much stronger than the metal-enzyme complex, so that the enzyme is displaced by EDTA and the radioactive peak is shifted to the right.

Figure 2 gives a plot of the enzymatic activity as a function of the concentration of the inhibitor, Ag^+ ion. The concentration of LDH-1 in these samples was approximately 3×10^{-7} *M*. The equation relating the enzymatic activity with the

Table I. Results of the Metal-Inhibition Studies of the LDH-1 Catalysis of Lactate to Pyruvate Reaction

LDH-1 or metals	Conc. in final reaction mixture (M)	A	Activity (U/mL)	% original activity	% reduction in activity
LDH-1	3.00×10^{-8}[a]	0.081 ±0.007	407 ±35		
Mn^{2+}	2.98×10^{-4}	0.079	395	97.1	2.9
Ni^{2+}	2.79×10^{-4}	0.076	380	93.4	6.6
SeO_4^{2-}	2.08×10^{-4}	0.076	380	93.4	6.6
Cd^{2+}	1.46×10^{-4}	0.069	345	84.8	15.2
Cu^{2+}	2.58×10^{-4}	0.039	195	47.9	52.1
Zn^{2+}	2.51×10^{-4}	0.038	190	46.7	53.3
VO_3^-	3.22×10^{-4}	0.028	140	34.4	65.6
Hg^{2+}	8.17×10^{-5}	0.013	65	16.0	84.0
Hg_2^{2+}	1.64×10^{-5}	0.011	55	13.5	86.5
Ag^+	6.70×10^{-6}	0	0	0.0	100.0
Ag^+	1.64×10^{-5}	0	0	0.0	100.0

[a] LDH concentration in the original stock solution was measured in microgram by the Bradford Coomassie Blue method; molecular weight of LDH = 140,000.

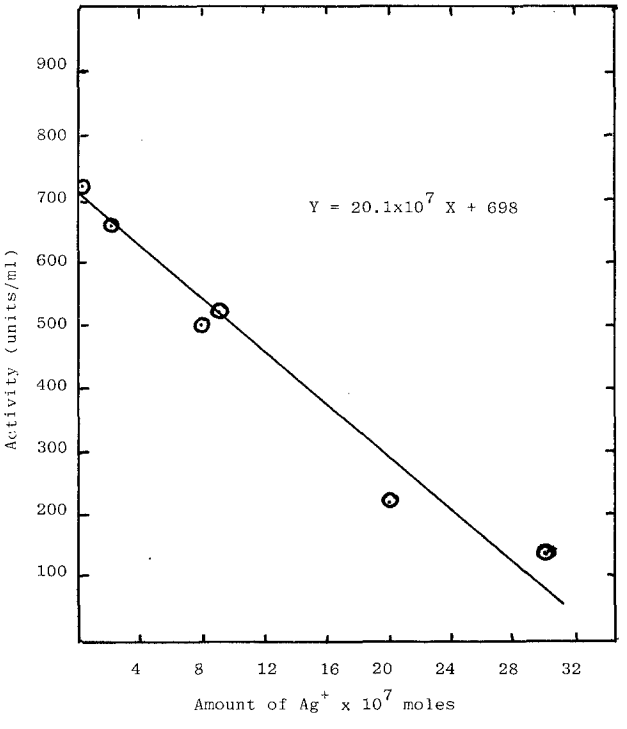

Fig. 2. A plot of the enzymatic activity of LDH-1 as a function of the concentration of the inhibitor Ag^+ ion.

Table II. Results of the Measurement of the Active Sites in LDH Using ^{110m}Ag as a Radiotracer Probe

Expt. no.	Mmol of LDH-1 reacted ($\times 10^6$)	Specific activity of Ag^+ ($\times 10^{-10}$)	Activity of separated LDH-1-Ag^+ ($\times 10^{-5}$)	No. of Mmol of Ag^+ bound to LDH-1 ($\times 10^5$)	Mmol of Ag^+ / Mmol of LDH-1
1	4.57	1.13	2.53	2.24	4.89
2	4.57	1.04	1.52	1.46	3.20
3	4.57	1.03	1.84	1.78	3.90
4	1.74	1.74	1.15	6.63	3.82
					Average = 3.95 + 0.70

concentration of silver ion obtained by the linear regression method is also given in this figure. The correlation coefficient ($r = 0.98$) shows that there is a good linear relation between the loss of enzymatic activity and the concentration of silver ion.

Table II shows the computational aspects of the determination of the silver-to-LDH mole ratio in the metal-bound LDH-1 or the number of active centers of LDH where Ag^+ is attached by the well-known "lock-and-key" mechanism. The average

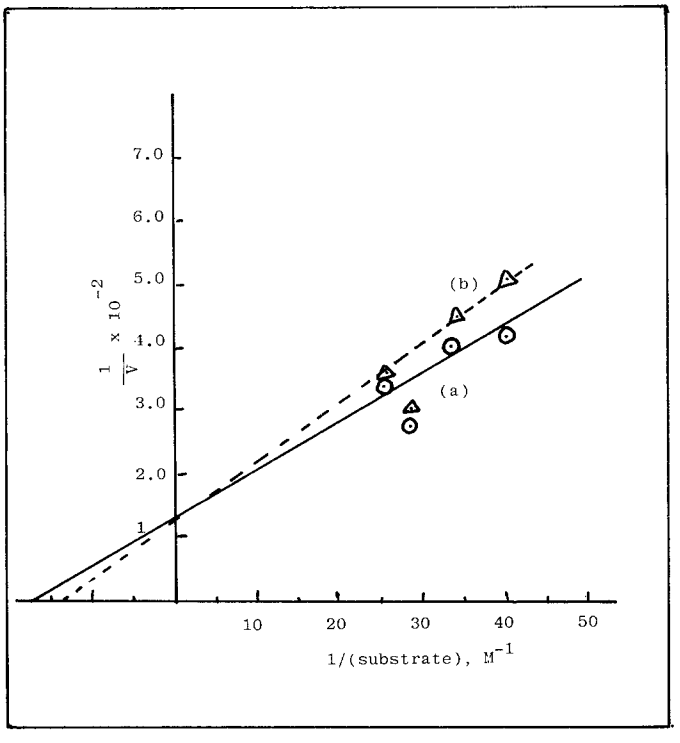

Fig. 3. Lineweaver-Burk plots of the reciprocal velocity vs. reciprocal substrate concentration (a) in the absence and (b) in the presence of Ag^+ ion.

number of silver ions bound to one molecule of LDH-1 obtained from four measurements is 3.95 ± 0.7. It is to be pointed out that the UV-visible spectra (not shown) of the pure LDH-1 sample and of the silver-bound LDH-1 were identical with λ_{max} at 296 nm.

Figure 3 illustrates the difference in the Lineweaver-Burk plots of the reciprocal velocity vs. the reciprocal substrate concentration in the presence and absence of Ag^+ ion. In both cases the concentrations of the enzyme, co-enzyme, total phosphate, and *p*H were kept constant. When the inhibitor (Ag^+) was used, its concentration was kept constant at 6.62×10^{-8} *M*. It can be observed from these plots that not only do the two straight-line graphs intersect at the *Y* axis, but also their slopes are significantly different. These straight-line graphs may be represented by the following equations:

$$1/V_i = K'_L/V'_f(L) + 1/V'_f \tag{2}$$

$$1/V_i = K'_L(1+(I)/K_I)/V'_f(L) + 1/V'_f \tag{3}$$

where V_i is the initial velocity, L is the concentration of lactate, I is the concentration of the inhibitor, Ag^+, K'_L the apparent Michaelis constant, V'_f the apparent maximum velocity, and K_I is the dissociation constant of the inhibitor-enzyme complex. These plots demonstrate that the interaction of Ag^+ ion with LDH-1 results in competitive inhibition.

4. DISCUSSION

There are several reports in the literature that the activation, inhibition, and denaturation of enzyme by metals or other ligands are due to conformational changes in the enzyme molecule (Jenehs, 1975; Vallee, 1981), due to the binding of metal ions to specific binding sites for certain metals (Bray, 1980) or due to the binding of the ligands with strong active sites (Murakami, 1982). Although lactate dehydrogenase enzyme is known to have active sites for interaction with substrates (Holbrook *et al.*, 1975), the enzyme activation or inhibition by metals has not been investigated so far. To the best of our knowledge this is the first attempt to use a radioactive metal probe for the determination of the number of active sites in the enzyme molecule. Separation of $*Ag^+$-LDH-1 complex from the unreacted $*Ag^+$ by size-exclusion chromatography coupled with the appearance of the metal-enzyme complex at the same location in the elution pattern suggests that Ag^+ ion forms a stable complex with the enzyme. However, EDTA was shown to displace the LDH from this complex forming a stronger EDTA-*Ag complex (Fig. 1c).

The reduction in the enzymatic activity in various degrees due to the presence of other metal ions in larger concentrations may be due to the presence of low-affinity sites for these metals or conformational equilibrium changes as suggested by Foreman and Niehaus (1985). However, the total loss of activity of LDH-1 due to the interaction with silver ion, at room temperature (25 ± 1°C), cannot be attributed to any conformational change, since there was no distortion of UV-visible spectrum of LDH-1 after the binding. The proportional loss of activity with an increase in the concentration of Ag^+ ion also suggests that all of the enzyme molecules should be completely saturated with Ag^+ ion at their binding sites for a total loss of the

activity. The saturation of all binding sites with Ag^+ ion will undoubtedly prevent the substrate molecules from binding to the active sites of the enzyme.

Radioactive tracer experiments lead to the conclusion that there are four binding sites on the tetramer, most probably one in each subunit. LDH enzyme contains neither any metal nor any disulfide bridges (Holbrook *et al.*, 1975). Many types of reagents have been used to identify residues in the active sites of this enzyme. The position of the substrate binding site on each subunit of LDH is known to lie between the binding site of the nicotinimide (coenzyme) and histidine-195 (Holbrook *et al.*, 1975). Histidine residue would be able to act as a source and sink of the proton in the dehydogenase reaction (Miller and Schwert, 1963). Tahenaka and Schwert (1956) have reported that each subunit has also one NAD^+ binding site. There is also evidence to suggest that pyruvate and lactate cannot bind to the enzyme in the absence of nucleotides as coenzymes. These facts will confirm that there are four active centers on LDH enzyme, one on each subunit, and that silver ion binds with the histidine-195 residue in each unit. Kinetic studies also reveal that the enzyme inhibition that resulted from the interaction of Ag^+ ion with LDH-1 is competitive inhibition in which the metal ion competes with the substrate for the binding sites. Therefore the inhibition of LDH catalysis of lactate-to-pyruvate reaction will conform to the "lock and key" model. This conclusion is also in agreement with the mechanism of catalysis of L-P reaction (Menon and Hunter, 1987).

5. SUMMARY

Lactate dehydrogenase enzyme inhibition studies with metals show that the enzymatic activity is reduced in various degrees by several metals at higher concentrations, but is destroyed completely by silver ion at relatively smaller concentration. Experiments with ^{110m}Ag-labeled Ag^+ demonstrate that Ag^+-LDH-1 complex is stable, but weaker than Ag-EDTA complex. A radiosilver probe was used to determine the number of active sites in the enzyme molecule. It was found that there are four active sites in the molecule, probably one on each subunit where the substrate is bound. This is in agreement with previous reports. Kinetic studies of the LDH catalysis of L-P reaction in the presence and absence of Ag^+ ion reveal that silver ion is involved in competitive inhibition and that the interaction of metal conforms to the "lock-and-key" model.

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