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The Metal Ion Status of Rat Liver Lactic Dehydrogenase

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Earlier experiments indicated that pure rat liver lactic dehydrogenase (LDH) was not strongly inhibited by various metal chelators. The present report supplies analytical data obtained by emission spectrographic analyses and demonstrates that this particular LDH contains only small and variable amounts of several metal ions, depending upon previous treatment. The effects of several metal chelating agents are described.

Experiments which provide information about the catalytic reaction sites of enzymes are of the greatest interest. In the case of highly purified dehydrogenases from various sources, the suggestion has been made by Vallee and coworkers[1,2] and by others[3–6] that a divalent cation at the reaction site may play an important role in the catalysis. This role is usually considered to involve chelation of coenzyme or substrate on the reaction site. If this suggestion is valid, one would expect that a pure dehydrogenase would show at least one mole of metal ion per mole of reaction sites. Examples of this kind of relationship have been reported for yeast alcohol dehydrogenase[7,8], horse liver alcohol dehydrogenase[9,10], rabbit muscle lactic dehydrogenase[11], and beef liver glutamic dehydrogenase. A contrary point of view with respect to a role for zinc in pig heart, rat and rabbit skeletal muscle lactic dehydrogenases was presented by Pfleiderer, Jeckel and Wieland[13]. These investigators found no appreciable inhibition of these particular dehydrogenases by metal chelating agents and no significant amounts of zinc after ashing.

It is, therefore, a matter of interest to examine another pure dehydrogenase and determine its metal ion status.

EXPERIMENTAL AND RESULTS

Emission spectrographic analyses

Rat liver LDH was prepared by either the procedure of Gibson *et al.*[14] or more recently by the modified method of Hsieh and Vestling[15]. Preparations of maximum specific activity were

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used. Davisson *et al.*[16] described the physico-chemical characterization of maximally pure LDH. The more convenient recent isolation method[15] involves (1) extraction of fresh or frozen rat liver with 0.5 M NaCl – 20 % ethanol; (2) ammonium sulfate fractionation; (3) ethanol fractionation; (4) carboxymethyl cellulose column fractionation with ionic strength gradient elution in the presence of 0.001 M 2-mercaptoethanol, pH 6.0; and (5) DEAE-cellulose column fractionation in 0.02 M phosphate – 0.001 M 2-mercaptoethanol, pH 7. From these steps recoveries of approximately 35 % of extractable LDH are realized, and the crystalline enzyme shows catalytic and physico-chemical properties identical to those of the pure LDH obtained by the Gibson[14] procedure.

In preparation for the metal content studies reagent grade ammonium sulfate and potassium chloride were recrystallized twice from 0.001 M Versene-Na_2 (disodium ethylenediamine tetraacetate). Glass-distilled, deionized water was used throughout, and glassware was soaked overnight in acid dichromate solution and thoroughly rinsed.

LDH of maximum purity prepared according to Gibson *et al.*[14] was treated as follows[17]: First it was salted out with purified ammonium sulfate and then dissolved in 0.1 M KCl and dialyzed on a rocking dialyzer at 4°C against 20 to 50 volumes of 0.1 M KCl with four changes during a 24-hour period. The first two dialyzing solutions were 0.1 M KCl – 10^{-4} M Versene-Na_2. After dialysis the LDH retained its maximum specific activity. This preparation was sent to Dr. B. L. Vallee, Biophysics Research Laboratory, Peter Bent Brigham Hospital, Boston, for emission spectrographic analysis by the porous cup spark excitation procedure. The authors are deeply indebted to Dr. Vallee for this assistance. The results, shown in Table 1, Column 1, indicate the presence of fractional molar quantities of Zn, Fe and Al and larger quantities of Mg and Ca (See Reference 1, p. 254.). Rat liver LDH has a molecular weight of 126 000[16] and shows two binding sites for NAD^+ or NADH per mole[18–21]. Accordingly, it would appear in the case of this particular LDH that Zn, Fe and Al are not involved in stoichiometric quantities at the catalytic site. The status of Mg and Ca will be clarified with the presentation of the data in Columns 2 and 3 in Table 1.

Recently pure LDH has been prepared by the Hsieh-Vestling procedure and subjected to the following steps. First, it was precipitated by the addition of purified ammonium sulfate and then dissolved in 0.05 M potassium phosphate-

Table 1. Rat liver lactic dehydrogenase. Emission spectrographic analyses.

	Moles of metal per mole of LDH (See text)		
Metal	1	2	3
Zn	0.37	0.35	0.37
Mg	4.03	7.05	1.35
Ca	10.0	10.0	1.32
Fe	0.38	Trace	Not detected
Al	0.41	Not detected	,, ,,
Sr	0.06	,, ,,	,, ,,
Ba	0.03	,, ,,	,, ,,
	Present but not determined: Na, K Not detected: Cd, Co, Cr, Mn, Mo, Ni, Pb, Sn, P Not determined: Cu	Trace: Cu Not detected: Na	Trace: Cu

0.001 M Versene-Na_2 – 0.001 M 2-mercaptoethanol, pH 7.6 and dialyzed for 12 hours at 4°C against three changes of 20–50 volumes of the same solution (rocking dialyzer used in all cases). Then it was dialyzed for 12 hours at 4°C against three changes of 0.1 M KCl. The dialyzed solution was assayed and then dried to constant weight in a platinum crucible (desiccator and 100°C oven). The weight of protein was obtained by subtracting the weight of KCl. The dried sample was submitted to Dr. S. W. Melsted and H. L. Motto, Department of Agronomy, University of Illinois, for emission spectrographic analysis. The authors are grateful for this assistance. The dried sample was ignited at 400°C overnight, and a rotating disk electrode with spark excitation was used. The results are presented in Table 1, Column 2. It is apparent that this LDH sample contained about the same quantities of Zn and Ca and more Mg than the earlier sample.

In order to see if further attempts to remove metal ions without loss of enzyme activity would be successful the following experiment was carried out: Pure LDH prepared by the Hsieh-Vestling method was precipitated by the addition of ammonium sulfate and dissolved in 0.05 M potassium phosphate – 0.001 M 2-mercaptoethanol, pH 7.6. The solution was dialyzed for 12 hours at 4°C against three changes of 20–50 volumes of 0.05 M potassium phosphate–10^{-4} M 1,10-phenanthroline, pH 7.6. The dialysis was continued for 12 hours against three changes of 0.05 M potassium phosphate – 0.001 M 2-mercaptoethanol–10^{-4} M Versene-Na_2, pH 7.6. A third 12-hour dialysis was carried out in the same way against 0.1 M KCl before submitting the sample to the Agronomy Department for analysis. Enzyme assays were varried out at each dialysis step. The specific activity of the pure LDH prior to dialysis was 9.15×10^4 μmoles NADH/min/mg protein; this value fell to 8.4×10^4 at the end of the dialysis. The total recovery of LDH during the dialysis steps was 92 %. The results shown in Table 1, Column 3, indicate a rather tenacious retention of a fractional molar quantity of Zn and sharply lowered levels of Mg and Ca. Again the conclusion must be drawn that Zn is probably not involved at the reaction site and that all three metals can be reduced to low levels of concentration without any effect on enzyme activity.

Terayama and Vestling[20] reported that several chelating agents were without marked effect on rat liver LDH activity. These agents included: Versene, 1,10-phenanthroline, 8-hydroxyquinoline, and α,α'-dipyridyl. Diethyldithiocarbamate, pyrophosphate, citrate, and salicylate were equally ineffective. Even after pre-incubation for one to three hours relatively slight inhibition – if any – was observed. In Table 2, data of this kind are presented. It is of interest to note that phenanthridine (5-aza-phenanthrene) is a very weak inhibitor; it is also not a chelator. This result is in contrast to that reported by Yielding and Tomkins[22] for glutamic dehydrogenase.

Effect of metal chelators

Terayama and Vestling[20] also noted that hydrosulfide ion inhibition of LDH could not be interpreted as evidence for metal ion participation in the catalysis, but rather that hydrosulfide ion inhibition could be reversed by any process which involved the removal or displacement of the SH^- ion in its apparent interaction with NAD^+ bound to the LDH reaction site.

Table 2. Effect of various agents on rat liver LDH Activity measured under standard assay conditions from the lactate side. Each cuvette contains: Sodium D,L-lactate, 0.11 M; sodium diethyl barbiturate, 0.03 M; NAD^+, 2×10^{-4}; LDH, approximately 10^{-9} M; pH 8.6; 25°C.

Reagent	Concentration M	Inhibition %
Versene-Na_2	10^{-2}	0
1,10-Phenanthroline	10^{-3}	5 to 10
,,	$10^{-2.5}$	10 to 20
Phenanthridine	10^{-4}	5 to 10
Diethyldithiocarbamate	10^{-2}	20
$ZnCl_2$	10^{-6}	0
,,	10^{-4}	10
,,	10^{-2}	90

Preliminary studies of the ultraviolet absorption and fluorescence spectra of pure LDH in combination with 1,10-phenanthroline and/or Zn^{++} support the general conclusion that the residual Zn^{++} which remains in the LDH molecule is available for interaction with 1,10-phenanthroline.

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