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Considerations in Evaluating the Zinc Content of Horse Liver Alcohol Dehydrogenase Preparations*

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ABSTRACT: Since horse liver alcohol dehydrogenase was first reported to contain 2 g-atoms of Zn/mol wt 73×10^3, the methods for purification of the enzyme and its molecular weight have been revised repeatedly. The specific activity of the product has increased substantially, resulting in uncertainties concerning the molar stoichiometry of metal and protein. The present paper reviews the results of zinc analyses of horse liver alcohol dehydrogenase preparations in this laboratory since the inception of work on this enzyme. The observed variations in zinc content are outside the limits of confidence of the methods employed for metal analysis. However, improvements in horse liver alcohol dehydrogenase isolation and characterization, including molecular weight determinations and recognition of the subunit structure and isoenzymes of horse liver alcohol dehydrogenase, all appear to bear on the variations in molar stoichiometry observed over the years. The zinc contents of recently obtained and uniformly treated samples of horse liver alcohol dehydrogenase vary from 3.1 to 4.3 g-atoms of Zn per mole of horse liver alcohol dehydrogenase, based on a molecular weight of 80×10^3. A variety of conditions alter both the zinc content and the catalytic activity of the enzyme. Only 2 g-atoms of Zn/mole of horse liver alcohol dehydrogenase appear to be directly related to enzymatic activity, as shown by selective removal of zinc from horse liver alcohol dehydrogenase, confirming the existence of two active-site zinc atoms.

Recent advances in protein chemistry have resulted in increased precision and sensitivity of methods employed in the determination of the physical, chemical, and functional properties of enzymes. Concurrently, procedures of high resolution have been developed for enzyme isolation and purification. When obtained by means of such improved methods, the numerical values for some of the properties of enzymes isolated by these refined procedures differ from those obtained on earlier preparations. Unfortunately, such reexaminations are often performed on some but not all of the pertinent parameters. This leaves open the question whether the changes observed originate from intrinsic differences of the materials examined, improved methodology, or both.

These evolving problems in analytical protein chemistry are well illustrated by LADH[1] first crystallized in 1948 (Bonnichsen and Wassén) and later shown to contain zinc (Theorell *et al.*, 1955; Vallee and Hoch, 1957). Since these early studies, procedures for the purification of LADH have been modified (Bonnichsen and Brink, 1955; Dalziel, 1958, 1960). Studies on LADH prepared by any of the procedures consistently show that it binds two molecules of reduced coenzyme, NADH (Theorell and Bonnichsen, 1951; Ehrenberg and Dalziel, 1958; Ulmer *et al.*, 1961), and requires zinc for catalytic function (Vallee and Hoch, 1957; Plane and Theorell, 1961; Yonetani, 1963). However, values for its total zinc content (Theorell *et al.*, 1955; Vallee and Hoch, 1957; Åkeson, 1964; Oppenheimer *et al.*, 1967), molecular weight (Ehrenberg and Dalziel, 1958; Hamburg, 1966; Drum *et al.*, 1967), and specific absorptivity at 280 mμ (Dalziel, 1958; Sigman, 1965; Hamburg, 1966; Taniguchi *et al.*, 1967) have varied, apparently depending upon the mode of preparation. Moreover, LADH has recently been shown to exist in several isoenzyme forms (Pietruszko *et al.*, 1966, 1969) and to have subunit structure (Drum *et al.*, 1967).

As a general convention, metal content of enzymes is usually expressed as a molar ratio, *i.e.*, gram-atoms of metal per mole of protein, rather than the ratio of mass of metal per gram of protein. Hence, alterations of the values of either metal content or molecular weight can also affect the molar stoichiometry. In order to delineate the role of zinc in LADH further, a more definitive identification of the factors affecting the zinc content of the molecule seemed necessary. Toward this end, this paper reviews the data on the zinc content

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[1] Abbreviation: LADH, horse liver alcohol dehydrogenase.

of the LADH preparations employed since studies of the enzyme were first initiated in this laboratory and compares them with the effect of a variety of conditions on the zinc content and activity of nine preparations recently obtained.

Materials and Methods

Review of Past Data on the Zinc Content of LADH. During the period 1955–1962, many different crystalline LADH samples were obtained for a variety of studies in this laboratory. Conditions for treatment of the enzyme samples prior to ashing were not uniform. Hence, in the course of these different investigations, the zinc content of such preparations was determined repeatedly by one or more procedures. The results of all such analyses were categorized according to the source, the identifying lot number of each sample, the number of aliquots for each sample analyzed, and the methods and number of zinc analyses performed.

Recent Enzyme Preparations. Four samples of crystalline LADH were obtained from the Boehringer-Mannheim Corp., prepared by a modification of the procedure described by Dalziel (1960), and four were furnished by the Worthington Biochemical Corp., prepared by the method of Bonnichsen and Brink (1955). Additionally, LADH was prepared in this laboratory from frozen horse liver by the latter method. The enzyme samples were stored at 4°, as suspensions of the washed crystals in 0.02 M sodium phosphate (pH 7) containing 10% ethanol.

Preparations of Samples for Analysis. Solutions of each of the crystalline LADH samples, approximately 1×10^{-4} M, were dialyzed against three changes of 50 volumes of 0.1 M Tris-Cl (pH 7.5), to remove phosphate ions, known to interfere with measurement of zinc by atomic absorption (Fuwa *et al.*, 1964). Specific activity was measured and, at this stage, zinc content was determined directly on a diluted aliquot of this sample by atomic absorption spectrophotometry.

The enzyme was then recrystallized from 0.02 M sodium phosphate (pH 6.8) containing 15% ethanol, at 4°. The crystals were harvested and dissolved, and solutions of $0.6\text{–}1.9 \times 10^{-4}$ M LADH were dialyzed for 21 hr against 100 volumes of 0.1 M sodium phosphate, pH 6.0, 4°. There was no loss of enzymatic activity under these conditions. Aliquots of each of these enzyme solutions were employed for the measurements of absorbance at 280 mμ, specific enzymatic activity, and protein dry weight, and zinc content was then measured again chemically, by diphenylthiocarbazone extraction, and spectrographically, by emission analysis.

Zinc Analyses. Three methods were employed, *i.e.*, chemical extraction using diphenylthiocarbazone, atomic absorption spectrometry, and emission spectrography. Primary working standard solutions of zinc for all analytical methods were prepared by dissolving weighed quantities of zinc rod (Johnson-Matthey and Co., Ltd.) in metal-free hydrochloric acid. To determine accuracy of the methods a Zn–Cu–Ni alloy, NBS sample 157-a, and a zinc spelter, NBS sample 109, both of certified zinc content, were dissolved in metal-free hydrochloric acid in the same manner as were the foregoing standards.

For the diphenylthiocarbazone extraction method (Vallee and Gibson, 1948), calibration curves were constructed from duplicate measurements of solutions containing 1, 3, and 5 μg of zinc diluted from primary standards. Triplicate samples, each containing 1.0–1.5 mg of enzyme, and dialysate blanks were pipetted into centrifuge tubes and the zinc was extracted by the trichloroacteic acid precipitation method previously described (Hoch and Vallee, 1949, 1953). By comparison with NBS standards, the accuracy of the method is 100 ± 2% for samples containing from 1 to 5 μg of zinc, and the standard deviation of the method is 2.5% (Vallee, 1954).

Specimens each containing 8–16 mg of enzyme were ashed and prepared for emission spectrography by methods previously described (Vallee, 1955). The ash was dissolved in 6 N HCl containing 7 ppm of vanadium as an internal standard. Intensity ratio working curves were constructed for each spectrographic plate by exposing four standards containing 1, 3, 10, and 30 μg of Zn per ml, each in triplicate, along with the enzyme samples. For zinc concentrations between 1 and 30 μg per ml, the coefficient of variation of this method varies from 7 to 10% (Thiers and Vallee, 1957), and the accuracy is 100 ± 7%.

Atomic absorption spectrometry was performed with equipment described previously (Fuwa and Vallee, 1963) using a Beckman burner and a 1.5 × 30 cm alundum absorption cell. Zinc in LADH was measured in the presence of 0.01 M Tris-Cl, previously shown not to interfere with the determination of zinc by atomic absorption (Fuwa *et al.*, 1964). For concentrations between 0.02 and 0.2 μg Zn per ml the standard deviation of this method is 2.5% and the acuracy 100 ± 3%.

Dry Weight Measurements. Protein weights were determined by triplicate trichloroacetic acid precipitation (Hoch and Vallee, 1953) of 8–16-mg quantities of LADH. The trichloroacetic acid precipitates were dried in tared borosilicate glass centrifuge tubes in a drying oven at 104° until minimum weight was reached, usually after 96–102 hr. For the various samples of LADH, specific absorptivities at 280 mμ were determined from such dry weights; the reproducibility of the method, expressed as ±2 std dev, is ±2%.

Assay for LADH Activity. Enzymatic activity was determined spectrophotometrically by measuring the rate of formation of NADH at 340 mμ, 23°. The assay mixture contained 5 μmoles of NAD^+, 50 μmoles of ethanol, and 50 μmoles of pyrophosphate (pH 8.8), in 3 ml, and the reaction was initiated by the addition of 0.1 ml of enzyme solution containing 7–12 μg of LADH. Specific activity is defined as the change in absorbance at 340 mμ/cm per min per mg of LADH; 2 std dev for the method is 3%.

Preparation and Purity of Reagents. Analytical grade chemicals were used throughout. All solutions were prepared with metal-free water obtained by slow passage through a mixed bed exchange resin (Dowex IR-120 and IRA-410) followed by distillation from a Pyrex glass still. Buffer solutions were extracted either at pH 5.7 or 7.5 with 0.003% diphenylthiocarbazone in CCl_4 until metal free and stored in acid-cleaned polyethylene bottles. Glassware and polyethylene containers were rendered metal free by soaking overnight in a 1:1 mixture of concentrated nitric and sulfuric acids, followed by rinsing with metal-free water before use. All dialyses were performed at 4° with cellulose casings treated as described previously (Hughes and Klotz, 1956). Volatile reagents such as trichloroacetic acid and ammonium hydroxide were freed of metals by distillation through a metal-free Pyrex condenser system.

NAD^+ (grade III) was purchased from the Sigma Chemical Co., St. Louis, Mo. Solutions (0.05 M, pH 7.5) were prepared

biweekly after which they were stored frozen until used. The purity was 98% as estimated from the known extinction at 260 mμ and the results of gel filtration over Sephadex G-10.

Molecular Weight. The molecular weight of LADH was determined by experiments performed in a Spinco Model E analytical ultracentrifuge, equipped with RTIC and a phase plate as the schlieren diaphragm. Details of the methods used in the centrifugation studies are reported elsewhere (J. H. Harrison, IV, T.-K. Li, J. L. Bethune, and B. L. Vallee, in preparation).

Chromatography and Electrophoresis. Analytical electrophoresis was performed on cellulose polyacetate strips using 0.02 M Tris-Cl buffer, pH 8.5, 23°. The strips were developed with an assay mixture containing phenazine methosulfate and nitro blue tetrazolium.

Chromatography of LADH solutions was performed on Whatman DEAE-cellulose (DE-23) supplied by H. Reeve Angel, Inc., Clifton, N. J. The isoenzyme C_3 of LADH was purified by a modification of the method of Pietruszko *et al.* (1966), employing a concentration gradient of Tris-Cl at pH 8.7, 4°.

Results

The Zinc Content of LADH (Past Preparations). During the period 1955–1962, ten different large-scale preparations of the enzyme, labeled 1–10 in Table I, were investigated in this laboratory. Separate aliquots, labeled a–d, from each of these samples were analyzed at different times. For each of these, duplicate microchemical analyses agreed well within the repeatability expected of the method. Further, these chemical analyses correlated well with emission spectrographic analyses performed concurrently. However, the zinc content of aliquots of the same sample analyzed *at different times* varied beyond the limits of reproducibility of either method. For example, the values of sample 4 varied from 2010 μg of Zn/g of protein in aliquot a to 3560 μg of Zn/g of protein in aliquot d. Similarly, variations of zinc content in *different successive preparations* are apparent (Table I).

In searching for the cause of such variation, the pertinence of a number of factors became apparent. The conditions to which different aliquots of the enzyme were exposed before analysis were not controlled and were not necessarily identical (*vide infra*). On the other hand, the increasing zinc content of *successive* preparations could have been the consequence of contamination or of an increase in the intrinsic zinc content of the enzyme incurred by changes in isolation procedure.

Zinc Content of LADH Treated Uniformly and Then Recrystallized (Recent Preparations). Such data and considerations led to the examination of the zinc content of nine enzyme preparations isolated between 1964 and 1966 by two different procedures, but prepared for analysis by identical techniques, thus eliminating possible variations due to time of storage and mode of handling (Table II). Four separate samples were obtained from each of two suppliers. Samples 11–14 (Boehringer-Mannheim Corp.) were prepared by a modification of the Dalziel method (1960) and 15–18 (Worthington Biochemical Corp.) by the Bonnichsen and Brink method (1955). Sample 19 was isolated in the laboratory using the latter method.

The zinc content of the different preparations varied markedly, ranging from about 2500 (in sample 12) to about 3500

TABLE I: Zinc Content of Horse Liver Alcohol Dehydrogenase Samples, 1955–1962.

Sample[a,b]	Zinc/Protein (μg/g)	
	Diphenylthiocarbazone	Emission Spectrography
1. W 5503	(a) 1630, 1820 (b) 1940, 1940 (c) 2140, 2290 (d) 2150, 2150	1640, 1770
2. W 5505	(a) 2680, 2470 (b) 3030, 3120 (c) 2810, 2640	2430, 2520 2940, 3270
3. W 5508	(a) 2780, 2950 (b) 2810, 2600	2480, 2620 2810, 2830
4. W 5512	(a) 2330, 2010 (b) 2810, 2920 (c) 2680, 2720 (d) 3560, 3510	
5. B 3127302	(a) 2580, 2610 (b) 2630, 2550	2620, 2730
6. B 3327203	(a) 2100, 2100 (b) 2720, 3020 (c) 2030, 2260	
7. B 5089208	(a) 3160, 3190 (b) 2920, 3020	
8. W 606	(a) 2630, 2840 (b) 2380, 2380 (c) 2530, 2630 (d) 2550, 2690	
9. B 6090109	(a) 3000, 3380 (b) 2460, 2440 (c) 2710, 2690 (d) 2460, 2570	
10. B 6291320	(a) 3030, 3050 (b) 2540, 2780 (c) 3280, 3020 (d) 2690, 2550	

[a] Samples obtained from the Worthington Biochemical Corp. are denoted by a prefix W, and samples from the Boehringer-Mannheim Corp. by the prefix B. Each number in the column headed "Sample" represents one preparation bearing a unique identification number. The samples are listed in chronological order as prepared and analyzed. Each letter designates a separate aliquot of the same sample, bearing the same identification number but received and analyzed separately. Based on these facts, identical analytical values would be expected for each of the aliquots from the same sample. Emission spectrographic analyses, performed originally to establish whether or not a functional metal was present, were performed later on only when contamination with other metals was suspected. [b] Samples 1–4 and 8 were isolated by the method of Bonnichsen and Brink (1955) and samples 5, 7, 9, and 10 according to a modification of the Dalziel procedure (1960). No information is available concerning the method of preparation of sample 6.

TABLE II: Zinc Content of Recent Horse Liver Alcohol Dehydrogenase Preparations.

Sample[a]	Zinc/Protein (μg/g)			Sp Absorptivity at 280 mμ (mg^{-1} cm^{-2})	Sp Enzymatic Act. (ΔA_{340} min^{-1} mg^{-1})
	Atomic Absorption	Diphenylthiocarbazone	Emission Spectrography		
11. B52426	3350	3130	3000	0.46	14.7
	3250	3140	3010		
	3150	3020			
12. B6225524	2530	2530	2550	0.41	14.4
	2530	2500	2600		
	2600	2420			
13. B6076327	2850	2800	2930	0.43	13.3
	2780	2750	2590		
	2850	2680			
14. B6256238	2600	2620	2900	0.43	13.0
	2720	2720	2800		
	2600	2550			
15. W6047	3380	3280	2790	0.50	14.2
	3490	3490	3320		
	3480	3540			
16. W6053	3240	3380	3300	0.47	12.9
	3240	3380	2850		
	3480	3180			
17. W6056	3260	3260	3240	0.47	13.4
	3260	3300	2930		
	3520	3500			
18. W6058	3520	3510	3200	0.49	14.8
	3540	3550	3370		
	3380	3510			
19. This laboratory	3180	3180	3040	0.47	14.2
	3180	3050	2700		
	3020	3120			

[a] Single aliquots of each sample were analyzed in triplicate or duplicate by three analytical methods; 2 standard deviations for the reproducibility of the methods are as follows: atomic absorption, 5%; diphenylthiocarbazone, 5%; emission spectrography, 15%; dry weight determinations for the quantities of protein used to determine specific absorptivity, 2%; specific enzymatic activity, 3%. All samples were prepared between 1964 and 1966.

μg per g (in sample 18). For each preparation, the excellent agreement among the three analytical methods seemed to preclude methodological considerations as the basis of the variation. Further, zinc content did not correlate with specific activity, suggesting that a fraction of the zinc content is not involved directly in function. However, the measured specific absorptivities at 280 mμ, ranging from 0.41 to 0.50 mg^{-1} cm^2, appeared to correlate with zinc content; the highest specific absorptivities coincided with the highest zinc content (Table II).

Compared as groups, the mean of the specific enzymatic activities of samples 11–14 does not differ significantly from that of samples 15–19, but the specific absorptivities and zinc contents of the former group tended to be lower than those of the latter.

Zinc Content and Absorptivity of Isoenzyme C_3. While such crystalline preparations of LADH do not contain enzymatically inactive proteins, they have been shown to be composed of electrophoretically distinct, multiple molecular forms (Pietruszko *et al.*, 1966; Theorell *et al.*, 1966). Isoenzyme C_3 (Pietruszko *et al.*, 1966), the predominant molecular form in these preparations, was purified from sample 14, which also contained significant quantities of isoenzymes C_4 and C_5 and a small amount of C_1 (Figure 1A). After chromatography on DEAE-cellulose, isoenzyme C_3 was virtually free of other isoenzymes (Figure 1B). The zinc content (2750 μg/g) of the recrystallized isoenzyme C_3, its specific absorptivity (0.43 mg^{-1} cm^2), and specific enzymatic activity (13.4) did not differ significantly from those of the parent mixture (sample 14), suggesting that these properties of the C_4 and C_5 isoenzymes are similar to those of C_3.

Effect of pH, Buffer, and Substrates on the Activity and Zinc Content of LADH. Data on earlier preparations (Table I) suggested that the conditions to which these enzymes were exposed while in solution and before analysis might have constituted a source of the variation in zinc content. Therefore the effects of pH, buffer composition, and enzyme concentration during dialysis and of metal binding agents upon the zinc content and enzymatic activity of LADH were examined.

When LADH was dialyzed against 0.1 M sodium phosphate

TABLE III: Effect of Added Zn^{2+} Ions on the Inactivation of LADH[a] by low pH (LADH, 1.2×10^{-5} M; Zn^{2+}, 1×10^{-4} M; 0.1 M Tris-acetate, 4°).

pH	Time (hr)	Sp Act.	
		$+Zn^{2+}$	$-Zn^{2+}$
5.0	0	14.4	14.4
	24	11.3	4.0
	72	7.8	1.3
5.5	0	14.4	14.4
	24	14.4	8.0
	72	13.2	3.9

[a] Sample 11 (B52426).

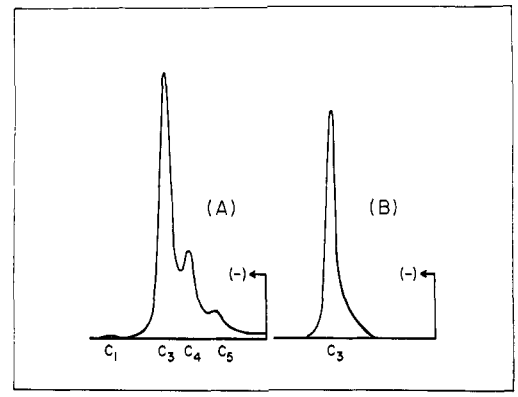

FIGURE 1: Analytical electrophoresis of liver alcohol dehydrogenase. The curves are densitometer scans of the cellulose polyacetate strips stained for activity before (A) and after (B) isolation of the C_3 isoenzyme from sample 14, Table II.

at pH values above pH 7.0, zinc content and activity remained stable for 80 hr. At pH values below 7.0, both activity and zinc content decreased progressively, but activity was lost more rapidly than was zinc (Figure 2). After 72 hr at pH 5.5, only 12% of the original activity remained, while zinc content had decreased to 820 μg/g, *i.e.*, 27% of the control value (Figure 2).

Furthermore, while the effect of pH is critical, the nature of the buffer employed constitutes an additional variable that influences LADH activity. After 7 hr of dialysis in Tris-acetate buffer at pH 5.5, enzymatic activity was 30% of the control (Table III), compared with the value of 12% for the identical experiment in phosphate buffer (Figure 2), emphasizing that the ionic species present also affect stability. Addition of 1×10^{-4} M Zn^{2+} to the dialysate greatly retards this loss of activity (Table III) suggesting that inactivation is due to the displacement of protein-bound zinc by hydrogen ions.

Chelating agents would be expected to accelerate loss of zinc from LADH engendered by hydrogen ions. Indeed, 1×10^{-2} M EDTA reduces both enzymatic acivity and zinc content of LADH to less than 10% of the control values after 65 hr of dialysis against 0.1 M Tris-Cl (pH 6.5) (Table IV). In contrast, the formation of ternary complexes of LADH with coenzymes and substrates should prevent loss of zinc from the active centers (Druyan and Vallee, 1964), and, hence, loss of activity (Yonetani and Theorell, 1962). Under conditions identical with those where EDTA removes zinc and activity is lost, the presence of 1×10^{-3} M NADH and 1 M ethanol fully prevents the loss of both.

Effect of LADH Concentration on Zinc Content at Low pH. During dialysis at low pH, both zinc content and activity of LADH approach minimum constant values (Figure 2). However, the particular value reached is strongly dependent upon enzyme concentration. After dialysis for 72 hr at pH 5.5, the zinc content of a 25-mg/ml solution of LADH remains unchanged at 3100 μg/g, while that of a solution containing 0.2 mg/ml of the same enzyme aliquot has declined to 400 μg/g

TABLE IV: Zinc Content and Activity of LADH[a] at Low pH in the Presence of EDTA and Substrates.[b]

Conditions	Sp Act.	Zinc Content (μg/g)[c]
Control	13.6	2800
LADH + EDTA (1×10^{-2} M)	0.9	250
LADH + EDTA (1×10^{-2} M), NADH (1×10^{-3} M), and ethanol (1.0 M)	13.8	2740

[a] Sample 13 (B6076327). [b] Activity and zinc content were measured after 65 hr of dialysis of 1.4×10^{-5} M LADH against 20 volumes of 0.1 M Tris-Cl, pH 6.5, 4°. [c] Zinc determinations by atomic absorption spectrometry.

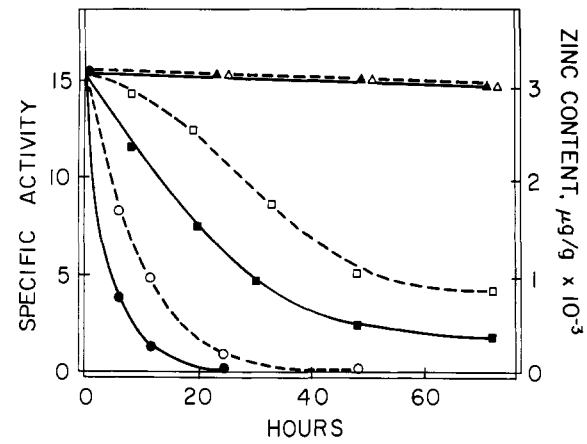

FIGURE 2: Effect of pH on the activity and zinc content of LADH. LADH (1.2×10^{-5} M) was dialyzed 3 days at pH 7.0 (▲ and △), 5.5 (■ and □), and 5.0 (● and ○) against 50 volumes of 0.1 M phosphate buffer at 4°. At the times indicated samples were withdrawn and analyzed for enzymatic activity (solid lines and filled symbols) and zinc content by the diphenylthiocarbazone method (dotted lines and unfilled symbols). Also performed but not shown were similar experiments at pH 6.0, 6.5, and 7.5. At all of the pH values studied both zinc content and enzymatic activity tended to approach an equilibrium value characteristic of that pH.

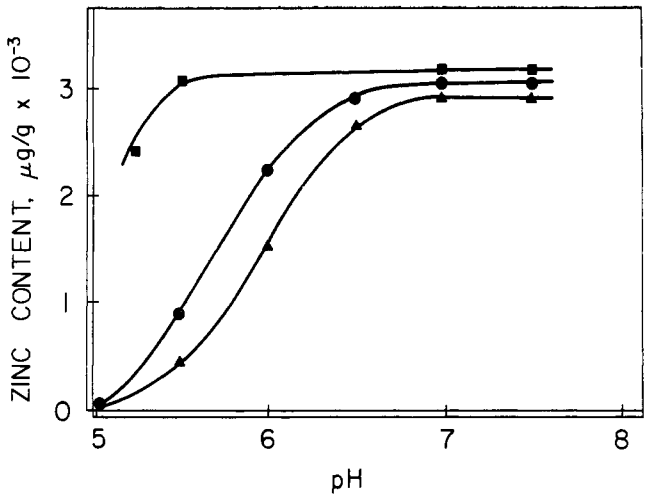

FIGURE 3: Effect of enzyme concentration on the zinc content of LADH at equilibrium. LADH at 25 mg/ml (■), 1 mg/ml (●), and 0.2 mg/ml (▲) was dialyzed against 50 volumes of 0.1 M phosphate buffer, 4° at pH 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 as in Figure 2. Values for the zinc content at equilibrium, reached within 72–96 hr, are plotted as a function of pH.

(Figure 3). Above pH 6.5, variation of the concentration of LADH during dialysis has little effect on zinc content. Variations in LADH concentration similarly affect specific enzymatic activity. At a given pH, a dilute solution of LADH loses activity more rapidly than does a concentrated one.

Removal of Zinc from LADH by Metal-Complexing Agents. In seeking a means for the selective removal of catalytically essential zinc atoms from LADH, the effects of a variety of metal complexing agents were studied. Samples of 1.2×10^{-5} M LADH were dialyzed for 72 hr against metal-free Tris-Cl buffer (pH 7.5) containing the agents listed in Table V in the concentrations indicated. At the end of this period, enzymatic activity of each sample was determined after 100-fold dilution into buffer free of complexing agents, thus lowering the actual concentration of the inhibitor in the assay by this factor. Zinc content was similarly determined after 30-fold dilution. When examined in this manner, none of the nitrogen- and/or oxygen-containing mono- and bidentate agents caused irreversible loss of activity by removing zinc.

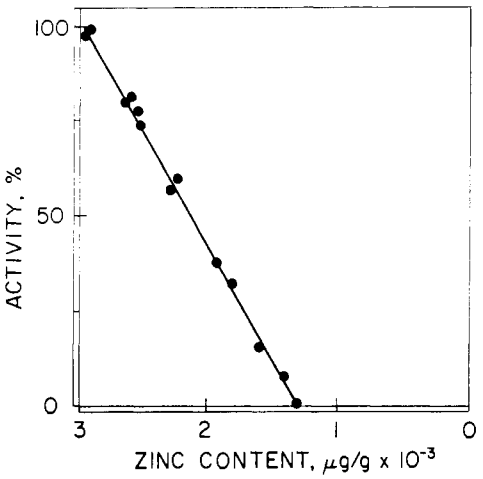

FIGURE 4: Zinc content and activity of LADH during inactivation by diethyldithiocarbamate. LADH (1.4×10^{-6}) was dialyzed against diethyldithiocarbamate (1×10^{-2}) in 0.1 M Tris-Cl, pH 7.5, at 4°. Aliquots of LADH were removed during the course of inactivation; activity and zinc content were determined. The results at each time of sampling are plotted in per cent of the activity of the control *vs.* the total zinc content in micrograms per gram of protein as determined by atomic absorption.

TABLE V: Zinc Content and Activity of LADH[a] in the Presence of Metal Complexing Agents.[b]

Agent (M)	Sp Act.	Zn Content (μg/g)
None	13.5	2850
1,10-Phenanthroline (1×10^{-3})	13.0	2850
2,2′-Bipyridine (1×10^{-3})	13.2	2900
Cyanide (1×10^{-3})	13.0	2850
8-Hydroxyquinoline-5-sulfonate (1×10^{-3})	13.6	2780
Imidazole (0.1)	13.7	2900
EDTA (0.5)	14.7	2850
2-Mercaptoethanol (0.1)[c]	1.8[d]	2850
Thioglycollate (0.1)[c]	5.2	2400
Cysteine (0.1)[c]	0	600
Diethyldithiocarbamate (1×10^{-2})[c]	0	1300

[a] Sample 13 (B6076327). [b] Experiments carried out by dialysis of 1.2×10^{-5} M LADH against 20 volumes of 0.1 M Tris-Cl, pH 7.5, 4°, containing the agent indicated. Activity and zinc content were measured at 72 hr; zinc determinations were made by atomic absorption spectrometry. [c] Deoxygenated and under nitrogen. [d] Inhibition fully reversible upon dilution.

Both 2-mercaptoethanol and thioglycollate inhibited LADH: inhibition by the former was fully reversible by further dilution, and zinc was not removed. The latter minimally affected zinc content, and the observed inhibition was partially reversible. Cysteine ultimately removed all of the zinc, concurrently precipitating the protein. Among the agents studied, the effect of diethyldithiocarbamate proved unique; it inactivated LADH irreversibly, but even prolonged dialysis against 0.1 M diethyldithiocarbamate did not remove more than 1650 μg of Zn/g (Figure 4), equivalent to 2 g-atoms of zinc/mol wt 80×10^3. Thus, a significant proportion of the zinc of LADH is not accessible to diethyldithiocarbamate, contrasting with hydrogen ions which effectively compete for all the zinc atoms. The decrease in enzymatic activity correlated directly with the removal of two zinc atoms (Figure 4), suggesting that diethyldithiocarbamate selectively removes only those which are catalytically essential.

Discussion

The earliest metal analyses of LADH indicated that preparations of the enzyme then available contained about 2 g-atoms of zinc/mol wt 73×10^3 (Theorell and Bonnichsen, 1951; Theorell *et al.*, 1955), or from 1.7 to 2.8, a mean of 2.1 g-atoms zinc/mole averaging the values of all spectrochemical, polarographic, and chemical analyses (Vallee and Hoch, 1957).

The numerical values for zinc obtained in both studies corresponded most closely to the 2 moles of NADH known to bind to the enzyme, a stoichiometry ascertained by independent measures (Theorell and Bonnichsen, 1951). The contribution of the molecular weight to these molar ratios is best appreciated in terms of Sund and Theorell's (1963) recalculation of these data, based on a molecular weight of 84×10^3 as redetermined subsequently (Ehrenberg, 1957; Ehrenberg and Dalziel, 1958), yielding values of 2.0–3.2, a mean of 2.4 g-atoms of zinc/mole. More recent analyses reporting values close to 4 g-atoms of zinc/mol wt 84×10^3 (Åkeson, 1964; Oppenheimer *et al.*, 1967) occasioned a detailed review of our data (Table I).

Three general considerations are germane to the evaluation of the metal content of biological samples: (1) features intrinsic to the analytical methods as expressed by their repeatability, precision and accuracy, (2) the selection of a representative sample, and (3) calculation and interpretation of the results in suitable terms of mass (Sandell and Elving, 1959). It would seem appropriate to examine the problem in these terms.

The evaluation of the methods and their contributions to possible incongruities are accomplished most readily both by the use of absolute standards of accuracy, provided by the National Bureau of Standards, and statistical approaches. The inherent characteristics of emission spectrography have proven valuable for the study of metalloenzymes, since the concentrations of many different elements may be measured simultaneously (Vallee, 1955). Over the range from 1 to 30 μg of Zn per ml the porous cup spark procedure employed in these studies has a coefficient of variation of 7–10%. Most of the variation arises from photography and densitometry; however, such precision has proven invariant in the course of continuous use of the method. Both the polarographic procedure previously employed (Kolthoff and Lingane, 1952) and the diphenylthiocarbazone method (Vallee and Gibson, 1948; Hoch and Vallee, 1949) have reproducibilities of $\pm 5\%$. Buffer constituents such as phosphate or carbonate commonly present in enzyme samples, and denaturing agents, such as urea, do not interfere with the dithizone method. The high specificity of the method, which requires small samples, is not easily surpassed. However, atomic absorption spectrometry is at least 10–100 times more sensitive than any of these procedures and, hence, the quantities of material required for analysis are even less, a consideration important in analyses of enzymes. For certain proteins, including LADH, atomic absorption measurements can be performed directly after dilution of protein solutions and without prior destruction of organic material, avoiding losses during ashing or contamination during sample preparations. The accuracy ($102 \pm 3\%$) and reproducibility (2 std dev = 5%) of atomic absorption are remarkable considering that absolute zinc concentrations as low as 0.001 μg/ml can be measured. Interferences are readily recognized and can be eliminated (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964). Accuracy of the methods was established by measurement of zinc in certified samples from the National Bureau of Standards.

Agreement between two or more of these independent analytical methods is shown in Tables I and II. On this basis the observed variations in zinc content of LADH cannot be attributed to analytic methodology. Hence, the sources of variations would appear to be intrinsic to the samples analyzed.

The selection of representative biological samples, while simple in principle, can present potential problems. As recently pointed out by Sober *et al.* (1965), few if any protein preparations satisfy all available criteria of purity. Different preparations of the same enzyme, isolated by the same procedure and homogeneous in all known respects, have proven to be inhomogeneous when the examination of new properties became possible. Thus, the selection of a sample truly representative of a given protein is often beset with uncertainty.

In the past, while different amounts of zinc were found in separate aliquots from the same LADH sample when these aliquots were prepared for analysis at different times (Table I), meaningful trends were not discerned, largely because of the frequency with which contamination had been encountered. Metal contamination of metalloenzymes has presented problems in evaluation of both compositional and activity data (Vallee *et al.*, 1956). Yeast alcohol dehydrogenase, for example, is inhibited by contaminating metal ions (Hoch *et al.*, 1960) and is, therefore, *activated* by their removal with 1,10-phenanthroline (Redetzki and Nowinski, 1957). In LADH, the presence of zinc in excess of 2 g-atoms/mole was thought to constitute extrinsic contamination; a possible structural role of zinc became apparent only on detection of the subunit structure of this and other enzymes toward the end of the period during which the analyses shown in Table I were performed.

This circumstance itself introduces new problems in terms of the purity of enzymes as discussed by Sober *et al.* (1965). Definition of the ultimate number of subunits of any protein presents itself as yet another problem in analytical technique, since the various approaches employed may yield different answers. Exposure of the enzyme samples to different conditions of dialysis prior to analysis might also have caused variations. The observed effects of pH, enzyme concentration, ionic environment, and complex formation with coenzymes and substrates on zinc content and enzymatic activity during dialysis (Figures 2 and 3 and Tables II and III) would support such assumptions.

It cannot be stated with certainty to what extent these and other factors may have contributed to the variation of the zinc content among the different preparations in Table I. However, when the zinc data obtained over a 7-year period are compared chronologically, a rising trend is unmistakable, both in the enzymes prepared by the Bonnichsen–Brink method (Table I, and samples 1–4 and 8) and by the Dalziel modification (Table I, and samples 5–7, 9, and 10). The zinc content of recent preparations is greater than 2500 μg/g (Table II), consistent with these earlier findings.

The presence of extraneous protein impurities or inactive enzyme may have lowered the zinc/LADH ratio in earlier preparations. The initial isolation proceure of LADH (Bonnichsen and Wassén, 1948) has been modified repeatedly (Bonnichsen and Brink, 1955; Dalziel, 1958, 1960). The specific activity of preparations assayed in 1955 under conditions identical with those employed in this study was half that of current preparations. The values of k_1', the rate constant for dissociation of the LADH–NADH complex, was found to be 1.6 sec^{-1} at pH 7.15 in 1955 (Theorell *et al.*, 1955). Under the same conditions, this constant obtained with enzyme prepared by an improved method was 2.9 and 2.8 sec^{-1} in 1960 (Dalziel, 1960). Apparently, there is no record of inactive protein impurities in LADH based on physicochemical criteria; activity and coenzyme binding have served as the operational gauges

of protein purity (Dalziel, 1957, 1958; Theorell *et al.*, 1955; Witter, 1960; Theorell and Yonetani, 1963). The chromatographic modification introduced by Dalziel (1958) demonstrated molecular inhomogeneity in crystalline LADH preparations, but the minor species were found to be as active enzymatically as the major component.

The choice of suitable units of measurement for metal content of proteins may become important when examining enzymes with subunit structure. Metal content of enzymes has been expressed conventionally in units of gram-atoms per mole. This value incorporates all methodological uncertainties already discussed as well as those of molecular weight determinations.

The molar ratio of zinc/LADH initially reported, *i.e.*, 1.7–2.8 g-atoms/mole (Theorell *et al.*, 1955; Vallee and Hoch, 1957), was based on the then accepted molecular weight of 73 $\times 10^3$. Using the same analytical values for zinc content but a molecular weight of 84 $\times 10^3$ (Ehrenberg and Dalziel, 1958) a ratio of 2.0–3.2 g-atoms/mole is obtained. Based on the most recently determined molecular weight, 80 $\times 10^3$ (Hamburg, 1966; Drum *et al.*, 1967; J. H. Harrison, IV, T.-K. Li, J. L. Bethune, and B. L. Vallee, in preparation), 1.9–3.1 g-atoms/mole of LADH would be calculated. Assuming this latter value for molecular weight, the molar ratio of zinc to enzyme reported in other studies (Åkeson, 1964; Oppenheimer *et al.*, 1967) ranges from 3.6 to 4.0 and that for the data in Table II ranges from 3.1 to 4.3 g-atoms per mole.

Crystalline enzyme samples recently prepared (Table II) all exhibit comparably high specific enzymatic activity and do not contain enzymatically inactive protein material which can be separated by gel filtration, electrophoresis, or chromatography. Hence, protein impurities do not appear to be the basis for the variation of their zinc content. However, the different values for the specific absorptivity of LADH observed here (Table II) have also been reported previously (Bonnichsen and Wassén, 1948; Dalziel, 1958; Sigman, 1965) although a correlation of zinc content with absorptivity (Table II) has not been documented. Such variations might relate to heterogeneity in protein composition and conformation or contamination with amino acids, peptides, and other chromophoric molecules, or combinations of these. Indeed, crystalline preparations of LADH have been shown to be contaminated with amino acids (Jörnvall, 1965) and to contain varying proportions of several isoenzymes (Pietruszko *et al.*, 1966; Theorell *et al.*, 1966). Furthermore, the purification procedures generally employed for isolation of LADH can remove selectively those isoenzymes which exhibit steroid dehydrogenase activity in favor of those with ethanol dehydrogenase activity (Pietruszko *et al.*, 1966; Theorell *et al.*, 1966).

Yet other evidence supports this line of reasoning. LADH is composed of subunits, and zinc participates in subunit interactions (Drum *et al.*, 1967). Nonidentical subunits have been identified and their hybridization accounts for the formation of at least three of the nine isoenzymes of LADH identified thus far (Pietruszko *et al.*, 1969).

As with other multichain proteins, the number of subunits detected in LADH has varied when different methods were employed (Drum *et al.*, 1967; Cheng *et al.*, 1968; Pietruszko *et al.*, 1969). Delineation of the number of subunits in LADH and the participation of zinc in their interactions present a problem in techniques of protein chemistry. The principles of analysis as detailed by Sandell and Elving (1959) (*vide supra*) would also seem to be applicable here. However, the compositional differences between nonidentical subunits have not yet been reported nor is the relationship of these three isoenzymes to the other isoenzymes present in LADH preparations established. Whether variations in isoenzyme composition significantly affect heterogeneity of zinc content and specific absorptivity of LADH preparations remains unresolved until these fundamental relationships can be discerned.[2]

Even the molar stoichiometry of zinc/protein of the purified isoenzyme C_3 may be nonintegral, indicating that sources unrelated to the LADH isoenzymes must be considered. In this regard, deviation from precise integral stoichiometry of gram-atoms of metal per mole of enzyme has been observed in other zinc-containing metalloenzymes with subunit structure. Thus, yeast alcohol dehydrogenase (mol wt 150 $\times 10^3$) may contain from 3.3 to 4.7 g-atoms of Zn/mole (Vallee and Hoch, 1955; Sund and Theorell, 1963). The zinc content of fully active *Escherichia coli* alkaline phosphatase, homogeneous by conventional physicochemical criteria, may vary from 2.1 to 3.8 g-atoms per mole, depending upon the mode of preparation (Plocke *et al.*, 1962; Harris and Coleman, 1968; Simpson *et al.*, 1968).

As suggested previously, such nonintegral stoichiometry may reflect the multiple roles which metals may serve in enzyme structure and function (Vallee, 1960). Thus, in contrast to enzymes consisting of a single peptide chain and containing a single metal atom serving largely a catalytic role (Vallee and Neurath, 1954; Lindskog, 1960; Folk *et al.*, 1960), metals in multichain enzymes may participate both in the catalytic process and in the maintenance of tertiary and/or quaternary structure. Electrostatic side-chain interactions, hydrogen bonding, or disulfide formation might conceivably replace metal atoms participating in structural maintenance, but without incurring appreciable changes in catalytic activity. In a sense, such replacements might be compared with the substitution of specific amino acid residues by others serving similar roles. Heterogeneity may then be apparent only by virtue of nonintegral values of amino acid residues detected by amino acid analysis. Similarly, variations in the content of "buried" or nonessential zinc atoms need not manifest different catalytic or electrophoretic properties.

The lack of correlation between zinc content and enzymatic activity (Table II) indicates that a proportion of the zinc atoms of LADH are not directly essential for function, and, as expected, at low pH, zinc content and activity of the enzyme do not correlate linearly (Figure 2). The effect of diethyldithiocarbamate on the zinc content and activity shows that two of the zinc atoms correlate directly with activity, consistent with previous hypotheses that only two of the zinc atoms are associated directly with the active enzymatic sites.

In contrast to the chemically reactive, "free" zinc atoms at the active enzymatic centers, the remainder of the zinc atoms in LADH, varying from 1.1 to 2.3 g-atoms per mole in the present preparations, are not involved directly in catalysis and have been referred to as "buried." They are thought to serve a structural role, as described elsewhere (Drum *et al.*, 1967),

[2] Sandler and McKay (1969) recently reported no differences in zinc content between C_3 and C_4 isoenzymes and a parent preparation of LADH containing 3.9 g-atoms of Zn/mole. However, the molecular weight and specific absorptivities of these preparations were not stated.

and their partial removal is compatible with continued full catalytic activity.

The methods available both for the isolation and physicochemical characterization of proteins place constraints upon the accuracy with which any given property is measured and the confidence with which it can be correlated meaningfully with others. As evidenced by the values for molecular weight, specific absorptivity at 280 mμ, zinc content, and enzymatic activity of LADH, the uncertainties concerning the molar stoichiometry of zinc in LADH which have arisen over the course of the past decade appear to be attributable largely to methodological improvements in enzyme isolation and characterization. It might be expected that along with continuing technological improvements and the isolation of the individual isoenzymes, reevaluation of these properties will be a continuing necessity. To this end, a new sedimentation technique, time lapse ultracentrifugation (Bethune and Simpson, 1969; J. L. Bethune and R. T. Simpson, in preparation), is presently being employed to study the role of zinc in the subunit interactions of LADH.

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